BLOOD CLOTTING AND ALLIED PROBLEMS

Transactions of the Third Conference
January 23 24 1950, New York, N Y

Edited by

JOSEPH E FLYNN

DEPARTMENT OF PATHOLOGY
COLLEGE OF PHYSICIANS & SURGEONS
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JOSIAH MACY, JR. FOUNDATION CONFERENCE PROGRAM

FRANK FREMONT SMITH

Medical Director

With the accelerating rate at which new knowledge is accumulating and with the increasing recognition that nature is of one piece it becomes evident that the continued isolation of the several branches of science from one another is a serious obstacle to scientific progress

Nowhere in science is the need for combined operations more evident than in medicine Today to be effective medical research and practice must embrace data from all the disciplines including nuclear physics at one end of the spectrum and cultural anthropology at the other for advances in one field are frequently dependent upon knowledge derived from quite another

Although the fertility of the multi discipline approach is thus recognized universities scientific societies and journals have not yet made adequate provision for channels of interdisciplinary communication

I am delighted to welcome you to the third meeting of this group on Blood Clotting and Allied Problems and to give you as briefly as possible an outline of what we hope to accomplish by these conferences

The Foundation is interested in furthering knowledge in this field as well as in investigating the broad aspects of problems of communication and integration which are important for the advancement of the whole of science It is our belief that scientific communications at scientific meetings and in the journals have been forced into a narrow mold in which logical sequence leading to inevitable conclusions is substituted for the much more flexible and often unpredictable processes by which scientific inquiry and the advance of knowledge actually take place All the creative the really exciting and interesting factors which are the soul and heart of science tend to be excluded today This unfortunately discourages individuals who have a creative and artistic turn of mind from entering the scientific field and creates in the minds of

us accept only data coming from those methods or disciplines with which we are familiar It is important that we do justice to the validity of data and methods from other disciplines On the semantic level the physical and biological sciences have little difficulty the medical psychiatric and social sciences can understand each other fairly well but to bridge the gap from the physical and biological sciences to the psychological and social is very difficult However in the study of man all of the sciences must meet In medicine which must be equally concerned with the psychological and social as with the biological and physical there is the greatest opportunity as well as necessity for mutual understanding among representatives of all the sciences I believe that the hope for a unification of science lies in the development of a *Science of Man* in which medicine must play a central role

In closing I want to say that the Conference Program is an experiment and that you are part of that experiment We hope that at these conferences you will feel the freedom inherent in the scientific method and will help us to improve our conference procedure

students and of the public a profound misunderstanding of the nature and processes of science

The Foundation s experience with the many research projects coming before it has led to the conviction that one of the greatest needs today is a re integration of science now artificially fragmented by the isolation of the several scientific disciplines and specialties Our Conference Program hopes to encourage this re integration and to give in the published transactions of our conferences a clear reflection of what takes place in the laboratory and what goes on in the minds of investigators than now appears in scientific literature

Our eight groups on aging blood clotting and allied problems blood pressure biological antioxidants cybernetics infancy and childhood liver injury and metabolic interrelations (formerly metabolic aspects of convalescence) which have been functioning for some years have held over 50 meetings in which more than 500 individuals have participated Recently five new conference groups have been organized on the following topics adrenal cortex renal function nerve impulse connective tissues and problems of consciousness

Each group will hold annual two day meetings for a period of five years It is our belief that only through continued association in an atmosphere of friendliness and mutual confidence can effective communication (exchange of ideas data methods and plans) across the barriers of the professions and specialties be promoted As a result of these meetings we have seen plans and ideas modified conclusions more clearly specified or placed in a broader perspective, and spontaneous collaboration take place between investigators working in different departments or in different universities

As a nucleus 15 scientists comprise the original group of members for any conference These are selected by the Chairman of the Conference in consultation with the Foundation Every effort is made to include representatives from all pertinent disciplines From time to time new members are added by the group to fill gaps in viewpoint or technique A limited number of guests are invited to attend each meeting but for the purpose of promoting full participation of all members and guests attendance at any meeting is limited to 25

A point which I should like to stress before closing is that between the disciplines there are real difficulties in communication — partly emotional and partly semantic Emotionally some of

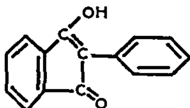
EXPERIMENTAL ASPECTS OF THE ANTICOAGULANT, PHENYLINDANDIONE

LOUIS B JAQUES
ERIC LEPP and ELLEN GORDON*

Department of Physiology University of Saskatchewan

Considerable interest has developed recently in compounds having the prothrombopenic action** of dicumarol but less permanent in their action and thus safer for clinical use Fantl in Australia[1] advocated the ethylidene analogue of dicumarol (3 3 ethylidene bis-4 hydroxy coumarin) Eriksen Jacobsen and Plum in Denmark[2] reported that dihydro dicumarol was effective Certain Czech and Swiss workers have introduced the acetic acid ethyl ester of dicumarol known commercially as pelentan and tromexan to be discussed by Dr Burke The French workers developed phenylindanedione***

In 1947 in the course of an investigation of the relation between structure and antivitamin K activity P Meunier C Mentzer and D Molho[3] reported that this drug phenylindanedione for which the structural formula is given below has a prothrombopenic action Because of its transitory action Meunier suggested that this might



2—PHENYLINDANEDIONE—1 3

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* From the Department of Physiology University of Saskatchewan Saskatoon Saskatchewan Aided by grants from the National Research Council (Canada) and Charles E Frosst & Co Montreal. The phenylindanedione (trade name—danilone) was prepared by Charles E Frosst & Co The authors acknowledge their indebtedness to Dr D F Moore Professor of Pathology University of Saskatchewan for pathological studies of the animals receiving phenylindanedione

** In agreement with recommendations arising from the discussion of this paper it is hereby stated that the terms "prothrombopenic action and "prothrombopenic agent" are used in this paper only in the sense of an increase in the accelerated clotting time (so-called prothrombin time") produced by certain chemical agents *in vivo* and for the agents producing this effect respectively

*** Optional spelling

rabbit brain powder The animals received the ordinary laboratory diet consisting chiefly of Fox chow cubes

Figure 1 shows the prothrombin time with 0 004 M calcium in rabbits receiving 50 mg P I D orally The solid line represents the values with undiluted plasma the dotted line represents those for 25% plasma Also shown is the response obtained in the same animal with 6 mg of dicumarol With both drugs it can be seen that the prothrombin time reached a peak value 24 hours after the anti coagulants were administered However after dicumarol the pro thrombin time took approximately 3 days to return to normal while recovery was complete in 14 hours following the use of P I D The prothrombin time after P I D as after dicumarol rises to a peak value following the tenth hour However in contrast to dicumarol P I D shows a distinctly different effect on the prothrombin time immediately after administration giving rise to an

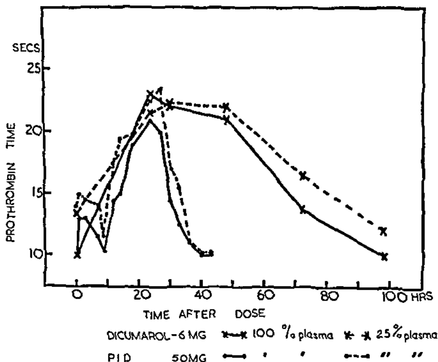


FIGURE 1

Effect of P I D and Dicumarol on Prothrombin Time in the Rabbit Rabbit #11 (weight 2 kg) Both drugs given orally at zero time Prothrombin time with 0 004 M Ca

be a valuable anticoagulant Also in 1947 Soulier and Gueguen[4] reported on the effects of single doses of phenylindanedione in animals and patients They reported that the drug was effective in lowering the prothrombin level. The doses required (10 20 mg per kg) were much higher than the effective dose of dicumarol However, the use of the drug was followed by rapid return of the prothrombin time to normal levels

The report by Meunier Mentzer and Molho led one of us (L B J) to ask Charles E Frosst & Co to prepare a sufficient amount of phenylindanedione for experimental tests Subsequently the drug will be referred to as P I D In the initial experiments single doses of the drug were given to rabbits and dogs and the prothrombin time determined After these experiments had been conducted, with largely negative results the report of Soulier and Gueguen came out To explain our lack of success it was pointed out by Dr Meunier* that he and his co-workers did not measure the action of the drug by the determination of the Quick prothrombin time Meunier used a photoelectric measurement Soulier used the whole blood single drop procedure These methods are more sensitive to small changes in prothrombin concentration than the Quick procedure and if their results were to be fairly tested it was necessary to use a method which was similarly sensitive to changes in prothrombin concentration This principle has been pointed out by Dr Link to this group on a previous occasion[5] The particular modification we adopted consisted in altering the calcium concentration In 1945 Dunlop & I [6] showed that after dicumarol the prothrombin time becomes very sensitive to changes in calcium concentration and hence this is one factor that may be used to make the system a more sensitive indicator of the effects of prothrombopenic agents We therefore used a concentration of 0 004 M calcium for determining prothrombin time The justification for this gambit (use of 0 004 M calcium) is the practical method by which we detected slight prothrombopenic activity in our initial experiments experiments which would otherwise have been reported as negative and the compound abandoned To check the results in the final experiments *determinations were simultaneously* made using 0 02 M calcium chloride Prothrombin times were determined on the plasma itself and also after dilution to 50% 25% and 12 5% as used by Link and others The thromboplastin used was a saline extraction at 53-55 C for 15 minutes of acetone dried

* (Personal communication to L B J)

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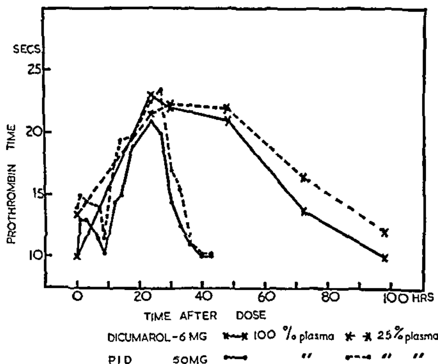


FIGURE 1

Effect of P I D and Dicumarol on Prothrombin Time in the Rabbit Rabbit #11 (weight 2 kg) Both drugs given orally at zero time Prothrombin time with 0 004 M Ca

elevated prothrombin time for two to three hours The effect passes off and is succeeded by the dicumarol effect This preliminary peak has been observed consistently As will be reported elsewhere P I D in comparable concentrations does not affect the prothrombin time when added to the blood *in vitro* so at present we have no explanation for this finding The rapidity of the effect of the drug on prothrombin time means that determinations of prothrombin time must be made at frequent intervals or the effect of the drug may be missed This occurred in preliminary experiments which were patterned after similar experiments with dicumarol.

Since the same peak prothrombin time was obtained with 6 mg of dicumarol as with 50 mg P I D this suggested that P I D has only about one tenth the potency of dicumarol To test this the effect of various dose levels of P I D on prothrombin time was studied (Table I) Examination of this table suggests that there is relatively no difference in the prothrombin time values obtained with different doses of P I D Thus Rabbit 11 showed the same response for doses of 25 mg and 100 mg By contrast doubling the dose of dicumarol at these levels approximately doubles the prothrombin time[7] With P I D the difference in response between different animals while not marked had a greater effect than differences in dosage level To our surprise in several experiments doses of 200 mg per kg and 20 mg per kg to these animals produced no observed effect on the prothrombin time While blood samples were not taken at sufficiently frequent intervals to enable one to say that no change had occurred it is surprising in view of the results obtained with intermediate doses that we did not observe some change in the prothrombin time

These results are not in agreement with the conclusions of Soulier and Gueguen that there was a direct relation between the dosage of P I D and the prothrombin time produced Inspection of the curves reported by Soulier and Gueguen however fails to demonstrate a clear direct proportionality between dosage and effect on prothrombin time

Wright Dr Jaques may I interrupt? In Table I Rabbit 19 has a peak prothrombin time value of 72 5 seconds — almost three times the value for the other rabbits Was this the only instance of unusual susceptibility?

Jaques Yes

Alexander Was P I D given orally or parenterally? I raise the question from the point of view of the possibility of variation in absorption of the drug

TABLE I

EFFECT OF SINGLE DOSES OF P I D ON PROTHROMBIN TIME

Subject	Dosage (in mg)	Preliminary Peak of Prothrombin Time (in sec)	Peak Value of Prothrombin Time (in sec)	Time to Reach Peak (Hours)	Time to Return to Normal (Hours)
Rabbit					
11	100	20	20 3	18	42
16	100	18 6	24 4	27	45
18	50	18 9	32.7	24	—
19	50	45 6	72.5	27	—
20	50	21 8	29 6	24	30
11	25	—	21 4	24	42
17	25	21.2	28 5	24	42
Dog					
Sa	50	20 5	—	—	33
Sp	50	23	30	26½	36
P	50	19.2	17.5	26½	36
Rabbit *dicumarol*					
16	3	—	38 0	46	105
11	3	—	26 9	24	89
17	3	—	24 8	42	90
18	3	—	32 8	44	92
Dog					
Sa	50	—	43 0	66	92

Prothrombin Time with 100% plasma and 0 004 M Ca normal prothrombin times were 10-12 secs for rabbits 9 13 secs for dogs

Jaques Orally except for a few experiments where P I D was given intravenously to several animals Such experiments showed that the drug was equally effective by vein and its action did not depend on the contents of the gastrointestinal tract as does sodium salicylate [8]

Returning to the main topic Figure 1 contrasts the difference in the rabbit between the administration of 6 mg of dicumarol and 50 mg of P I D It is obvious that P I D is a weak prothrombopenic agent compared to dicumarol but that recovery of the prothrombin time is faster following the administration of P I D than of dicumarol

EFFECT OF REPEATED DOSES OF P I D

In view of the transitory nature of the effect of P I D the effect on prothombin time of repeated doses of the drug was studied

elevated prothrombin time for two to three hours The effect passes off and is succeeded by the dicumarol effect This preliminary peak has been observed consistently As will be reported elsewhere, P I D in comparable concentrations does not affect the prothrombin time when added to the blood *in vitro,* so at present we have no explanation for this finding The rapidity of the effect of the drug on prothrombin time means that determinations of prothrombin time must be made at frequent intervals or the effect of the drug may be missed This occurred in preliminary experiments which were patterned after similar experiments with dicumarol

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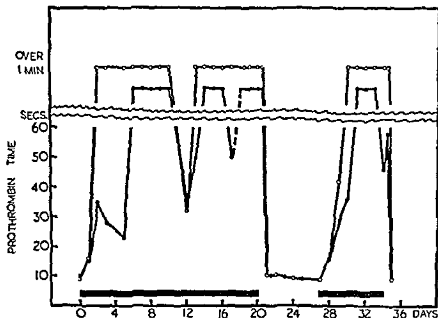


FIGURE 2

Effect of Repeated Doses of P I D on Prothrombin Time of Dogs Dog Sp 7 1 kg 8 mg per kg given every 8 hours o———o Prothrombin Times with 0 004 M Ca ●———● with 0 02 M Ca

day did not show much variation indicating that the failure to observe an increased prothrombin time with these single doses every 24 hours was not due to rapid changes in the prothrombin time occurring during the course of each 24 hour period

While the dose levels shown in Figures 2 and 3 both give the same extremely prolonged prothrombin time when administered 3 times a day there is a suggestion in Figure 3 that with the smaller dosage maintenance of prolonged prothrombin times was not as consistent since marked fluctuations occurred in the value and prothrombin time dropped occasionally for no apparent reason to the range of 20 to 30 seconds Two factors appeared to be con tributory One factor was failure on the part of the dog to ingest the drug in spite of all precautions to ensure this Thus on the morning of the 5th day the capsule was found in the cage of dog Pe (Figure 3) and it is suggestive that this failure to ingest the drug on the previous evening coincides with the low prothrombin times on the morning and evening of that day This same factor may also be operative and may explain some of the irregularities

Preliminary experiments indicated that it was advisable to administer the drug every 8 hours Various amounts of the drug were given orally to dogs and rabbits every 8 hours for periods of 4 to 46 days As shown in Figure 2, repeated administration of P I D to dogs resulted in a prolonged prothrombin time Eight milligrams per kilogram every 8 hours raised the prothrombin time one minute

Wright Dr Jaques when you kept the prothrombin time over 3 minutes for long periods of time were there any hemorrhagic manifestations?

Jaques They were very rare There were two dogs that did show hemorrhagic manifestations and I will discuss them later

Allen Was there any difference between the susceptibility to hemorrhage at a given prothrombin time with P I D as compared with dicumarol at the same levels or at the same time?

Jaques We have the impression that susceptibility is much less As indicated in Figure 2 after 20 days the drug was withdrawn and within 24 hours the prothrombin time was normal This appears to be the most valuable property of the drug We have found recovery so rapid that failure to ingest a single dose of P I D is reflected in the prothrombin time This may be responsible for irregularities observed in the Figure Figure 2 likewise shows the results obtained when the prothrombin times were determined with 0 004 M calcium and with the more concentrated solutions generally used Due to the insensitivity of the system for slight decreases in prothrombin the action of P I D is apparently delayed when the prothrombin time is measured with the higher calcium concentration

Since an amount of the drug which had little effect on prothrombin time when given in a single dose had such a marked effect when the dose was repeated the effect of a number of injections in the 24 hour period was studied (Figure 3) Twenty five milligrams per kilogram per 24 hours were given in 3 divided doses for 7 days then a single dose each 24 hours and finally in 3 divided doses for an additional 30 days It can be seen that a marked rise in the prothrombin time occurred on the third day Thereafter these high values of the prothrombin time were maintained with some fluctuations On the 8th day when the dose of 8 mg per kg every 8 hours was replaced by a dose of 25 mg per kg every 24 hours the prothrombin time fell almost to normal (ranging from 12 to 20 seconds) On the 14th day the blood samples were taken every 4 hours to determine if there were marked variations in the prothrombin time during one day Values during the course of the

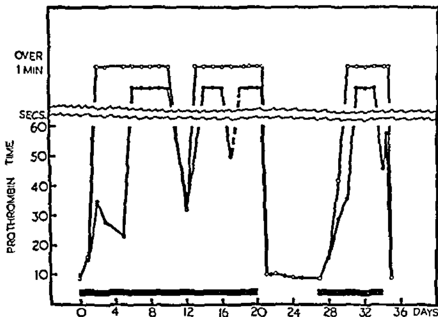


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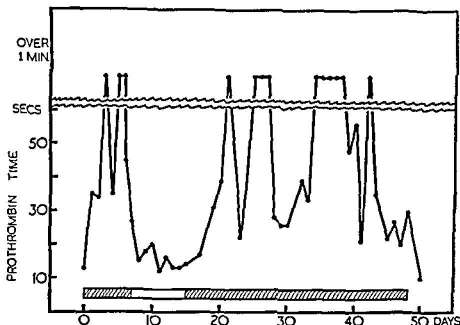


FIGURE 3

Comparison of Effect of Divided Doses of P I D on Prothrombin Time

[blank box] 25 mg per kg per 24 hours in single dose

[hatched box] 25 mg per kg per 24 hours divided into three equal doses Dog Pe 6 8 kg Ca concentration – 0 004 M

during the second period of 8 hour dosage As in this period the blood samples were taken at much greater intervals This gave the appearance of much longer periods of decreased prothrombin time and greater fluctuations (*e g for the 23rd day) The fact that even* missing one capsule per 8 hour period affected the prothrombin time response indicates the rapid action of this drug compared to dicumarol Another operative factor may have been the protein level of the diet

The following is something about which I was not going to say anything until I read the paper by Foley and Wright [9] *Between the* 24th and 30th day there was a period when the prothrombin time stayed at a very low value During this interval meat was fed to the animals Following removal of meat from the diet the prothrombin time rose to high levels again On the 44th and following days meat was again added to the diet and again the prothrombin time fell to a low value Foley and Wright[9] *have already suggested* that protein intake affects the effectiveness of dicumarol

In Figure 4 is shown the prothrombin time response when P I D was administered to rabbits (50 mg per kg were given daily in 3 divided doses) As with the dogs a hypoprothrombinemia was produced The animals were maintained on the drug for 6 days The prothrombin time reached the normal value 48 hours after the last dose of drug The quantitative similarity in the response of the two species is remarkable Some of the animals studied received simultaneously the same weight of vitamin K (2 methyl 1 4 naphthoquinone) as of P I D and this was repeated at several dose levels of P I D As shown in Figure 4 there was no difference in response between the animal receiving P I D and the one receiving P I D with vitamin K Hence in contrast to results obtained in similar experiments with vitamin K and dicumarol the giving a weight of vitamin K equal to the weight of this prothrombopenic

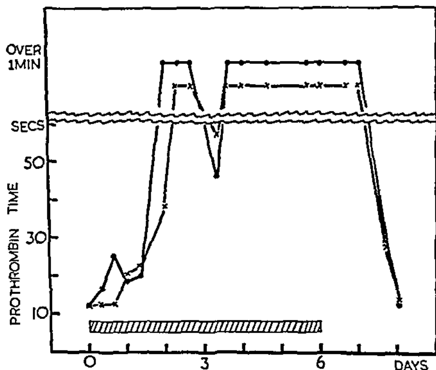


FIGURE 4

Effect of P I D and P I D with vitamin K in Rabbits 50 mg per kg of P I.D and 50 mg per kg of vitamin K per day divided in three doses x———x Rabbit 16 P I D ●———● Rabbit 11 P I D plus vitamin K

agent did not affect the change in prothrombin time in any way This experiment has been repeated with dogs with identical results Soulier and Gueguen reported that vitamin K had no effect on the increased prothrombin time produced by a single dose of P I D

Allen Did you try larger doses of vitamin K?

Jaques We gave large doses of vitamin K We were giving 50 mg per kg of P I D and 50 mg of vitamin K per kg This is very nearly the amount reported to be toxic

Barker Was the vitamin K given orally or intravenously?

Jaques Orally

Olwin What is the toxic dose in rabbits?

Jaques We searched the literature I don t know the exact value for the rabbit but the value found for toxic levels in some other species was of the order of 50 mg to 100 mg per kg Hence we did not care to press the experiment any farther

Figure 5 illustrates an experiment where repeated small doses of P I D given at short intervals (8 hours) were effective in causing an increase in prothrombin time Doses of 2 mg and 1 mg per kg given every 8 hours to rabbits resulted in the prothrombin *time being increased to values between 20 and 40 seconds* One sample from one animal gave a prothrombin time of 165 seconds but this was not maintained Variation during the maintenance period suggested that some fluctuation in the prothrombin time occurred between dosage periods With these low dosages as with the high dosages the same rapid rise in prothrombin time at the beginning of the experiment and the same rapid return to normal on stopping the drug was observed The effect of varying dosage has not been studied further as it was considered of greater significance to determine the consistency of the response for the dosage levels selected Repeated experiments with dogs and rabbits using the 8 3 mg and 16 7 mg per kg doses (approximately 25 mg and 50 mg per kg per day) consistently gave the response shown in Figures 2 3 and 4 Since these dose levels give prothrombin time of what practically can be considered infinity (over 10 minutes) they represent the upper limit of dosage Presumably there is a lower limit of dosage below which the drug fails to affect the prothrombin time The values shown in Figure 5 indicate that this level is approximately 1 mg per kg It appears that in rabbits there is a minimum level between which the prothrombin time response does not increase with dosage but above which the response increases very rapidly

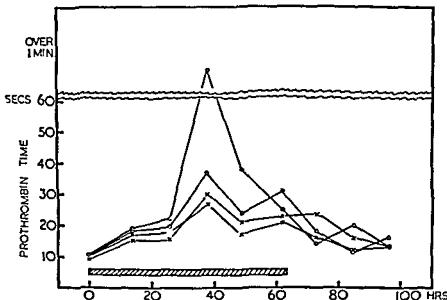


FIGURE 5

Effect of small doses of P.I D on Prothrombin Time x———x 1 mg. per kg every 8 hours o———o 2 mg per kg every 8 hours Rabbits.

TOXICITY OF P I D

All animals receiving the drug were studied for symptoms of toxicity With dicumarol or any other drug for that matter having a marked pharmacological action one must distinguish the effects of overdosage of the drug (in this case the effects of a marked decrease in prothrombin time) from side effects unrelated to the desired action of the drug A prothrombin time of infinity (over 10 minutes) presumably indicates an undesirable degree of hypocoagulability of the blood and therefore represents overdosage Hemorrhage was a definite phenomenon with our animals when the prothrombin time became infinity Hemostatis could be satis factorily produced by ordinary pressure if a 25-gauge needle were used for the blood sample However with an 18 gauge needle hemostasis was very difficult and prolonged bleeding from the veins tended to occur There was no effect observed on the red cell white cell or platelet count after continuous administration of P I D No change was observed in the hematocrit value and dif ferential white cell count which were determined at intervals on

some of the animals receiving the drug Soulier and Gueguen reported renal damage with very large doses of the drug Therefore on all occasions when urine was collected it was examined microscopically with almost uniformly negative results

Two animals maintained on the drug for a long period of time with very prolonged prothrombin times showed interesting hemorrhagic episodes One dog after 23 days with a prothrombin time of infinity, developed a severe hematoma associated with a superficial infection On stopping the drug the hematoma was rapidly reabsorbed The second dog after two weeks on P I D received a second dose of antidistemper serum Following this he showed a marked increased tendency to bleed with many small hematomas

Allen How soon did the hemorrhagic tendency occur after the administration of the antidistemper serum?

Jaques The dog received the serum at 10 a m No immediate effects were observed but at 4 30 p m when the dose of drug was given he appeared listless sleepy and unable to walk The veins of all legs were swollen tender to touch and bled very easily There was prolonged bleeding from small needle punctures, numerous small hematomas along leg veins and a large soft swelling in right axilla (probably 30 cc 50 cc of blood) This was 6½ hours after the antidistemper serum

As a result he quickly showed a severe anemia and thrombocytopenia leading to prostration He showed a typical picture of severe sweet clover disease or dicumarol poisoning However on discontinuing the drug without any transfusions or vitamin K complete recovery occurred in 65 hours In my experience dogs showing as severe a hemorrhagic syndrome after dicumarol do not recover without transfusions

Several dogs were maintained for 50 days on the drug with prothrombin times of infinity for most of the period yet manifested no hemorrhagic condition at any time Two rabbits and one of the dogs studied died during the investigation and a similar number were sacrificed We are indebted to Dr D F Moore for studying these animals for anatomical lesions No histological lesions ascribable to the P I D were observed in heart lungs liver or intestine In the kidney a mild fatty degeneration particularly noticeable in the limbs of Henle s loops was observed These animals had received from 0 5 gm 3 0 gm per kg of P I D over a period of some months

Possible long term toxicity of P I D was further studied in mice Eighteen mice were kept for 3 months on a diet containing P I D

The food was weighed daily and adjusted so that 9 of the mice received 25 mg P I D per kg each day while the other 9 mice similarly received 3 mg per kg daily for this period The controls were 9 mice receiving no P I D The mice were killed at intervals and the tissues studied by Dr D F Moore Professor of Pathology One mouse in 9 which received 25 mg per kg daily showed slight fatty degeneration in the liver This mouse had been on P I D only for 8 days Of the mice receiving 3 mg per kg daily the animals sacrificed at 3 7 and 8 days showed fatty degeneration in the liver 2 mice showed this effect in the kidney and 1 in the heart However the remaining 6 animals had no demonstrable lesions In view of the renal lesions observed in the dog it is interesting that no renal lesions were observed in mice receiving continued heavy doses of P I D However fatty degeneration in the kidneys was observed in two of the mice which received smaller dosages No anatomical lesions were observed in the control series

CONCLUSION

Phenylindanedione given in single doses was a weak prothrombopenic agent in rabbits and dogs However on repeated administration every 8 hours it was an effective prothrombopenic drug Repeated administration of 8 mg and 16 mg per kg resulted in a prothrombin time over 1 minute which was maintained as long as 50 days Cessation of administration of the drug resulted in a prompt fall in the prothrombin time to normal values within 40 hours Doses of 1 mg and 2 mg per kg every 8 hours maintained the prothrombin time of rabbits at twice the normal value While a hemorrhagic condition could be demonstrated with overdosage of the drug this could be easily controlled by stopping the administration of the drug Prolonged continued administration of very large doses of phenylindanedione to dogs rabbits and mice for periods up to 3 months resulted in damage to liver or kidney in a small percentage of the animals

The greater rapidity of action and recovery with this drug compared to dicumarol and the lack of toxicity indicates that this drug may be of value as a prothrombopenic agent

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DISCUSSION

Brambel We were very much interested to learn that phenylindanedione had anticoagulant properties However scattered local reports indicated that this compound was used in commercial production of an insecticide Work had to be stopped because of its toxicity to personnel since too many hemorrhagic hazards were encountered Thus it appears that drugs used clinically as anticoagulants are not only proving to be good raticides but insecticides as well

Is the mechanism of action of phenylindanedione the same as dicumarol? You mentioned in one of your papers that Factor V may be inhibited If so such observations would be most interesting

Jaques At the present time a sufficiently adequate investigation of the change in the clotting system has not yet been made I can give the results of the few determinations that have been made Dr A J Richards working in my department found with dogs a marked decrease in prothrombin concentration and a less marked decrease in the Factor V activity On the other hand Dr Paul Owren of Oslo Norway kindly sent me the results that he obtained with the first patient to whom he gave phenylindanedione and he observed a marked decrease in prothrombin concentration with no change in Factor V A sample of dog plasma after the administration of P I D was sent to Dr Seegers who reported a very marked fall in prothrombin activity It was actually less than 10 units per cc with a moderate fall 43 units per cc in Ac globulin values Dr Blaustein is at present completing preparations to do Ac globulin and prothrombin titers on patients receiving P I D We expect very shortly to have some satisfactory data on this matter

Faye Olwin and Ware report that dicumarol affects both prothrombin and Ac-globulin It affects the former much more and dissociation of the two effects has been observed with sustained doses I suspect — although I have no right to suspect without some experimental evidence — that we may find a similar situation with P I D It may affect both prothrombin and Factor V or Ac globulin Perhaps a dissociation of the two effects is possible This would explain the discrepancies that have already appeared even in our preliminary determinations

Brambel I have another comment to make if I may It may follow Dr Blaustein s presentation better We picked what we thought were "normal" patients for study of the effects of this drug Out of a group of 10 patients we encountered one who had a marked prolongation of the Quick prothrombin time After a 100 mg dose a 30-second prothrombin clotting time was found to persist for 5 days On reviewing the record of this patient a previous history of liver disease was noted The clinical history of the patient especially with regard to hepatic involvement should be considered This patient did not show the same phenomenon when dicumarol was administered

Barker Were parallel determinations of prothrombin by the one stage and two-stage methods done on any of the animals that received P I D and if so was there any discrepancy in the results particularly during initial period of increasing prothrombin deficiency?

Jaques Such studies have not been done largely because Dr Blaustein has undertaken this in connection with his clinical investigations

Flynn Dr Jaques how does P I D compare with dicumarol on a molar basis per minimum maintenance dose?

Jaques I don t believe you can carry out the comparison of the two drugs so exactly as to decide between them whether the effects are on a molar basis or a weight basis I think I will be able to clarify this when I give my other paper this afternoon

Quick I would like to make a comment on calcium I think Dr Jaques findings are interesting because they are just the opposite of what is observed in the hypoprothrombinemia of vitamin K deficiency and after dicumarol In those conditions more calcium is required in order to obtain the minimum prothrombin time In the results just reported apparently less calcium is required to get the minimum prothrombin time In regard to the labile factor I might state that Dr Stefanini and I found that it was not reduced

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Jaques May I reply to that? The fault lies not in ourselves We use the term prothrombopenic agent in the sense of a drug that affects the prothrombin time Any criticism should be directed at the workers who designated the accelerated clotting time as the "prothrombin time"

Alexander Might it not be more advantageous to refer to over all prothrombic activity rather than to any specific component involved in the prothrombin time?

Jaques I dont agree with Dr Alexanders suggestion because I dont think we can go as far as that If we are not allowed to use the term "prothrombopenic agent" then I think we have to use the term "an agent which increases the prothrombin time of animals and patients" and this is rather verbose

Alexander From the data presented I am not at all sure that fibrinogen was not affected

Jaques That may be I believe this is a matter for further investigation May I have the privilege of defining my terms? I am using the term "prothrombopenic agent" to refer to an agent causing an increase in the "prothrombin time"

Overman In Figure 1 some of the prothrombin times were lower with diluted plasma (broken line) than with whole plasma (solid line) Did you do antithrombin determinations on the diluted and whole plasma?

Jaques No antithrombin determinations were done

Tocantins Might the lower prothrombin times with diluted plasma have been due partly to the fact that a low suboptimal molarity of calcium was used in the undiluted plasma while the same molarity of calcium was used in the diluted one? Thus the calcium in the diluted plasma would be closer to an optimal level and the clotting time would be shorter

Jaques That may be the explanation

Allen I was curious about the two animals that showed hemorrhage It seems to me they may have one thing in common One of the animals it is stated had an infection and the other animal had received hyperimmune antidistemper serum Is that correct?

Jaques Yes

Allen I have three questions I would like to ask First do you believe that infection and possibly an anaphylactoid reaction from the antisera could have explained your hemorrhage when severe prothrombin deficiency was present? Second does P I D in any way affect a resolution of the clot? Third do you know how this

by dicumarol May I ask Dr Jaques why he did not use the two stage method?

Jaques I am afraid that with regard to the calcium Dr Quick you misunderstood the point We selected a calcium concentration that was not optimal because in the first experiments we were unable to find any effect with this drug We suspected that the decrease in the concentration of prothrombin was not very great so that when we used optimal calcium we were not able to detect the change in prothrombin Hence we purposely shifted to another calcium concentration which gave us a more sensitive test We just picked out calcium because it was something we had worked with before There is no particular reason for saying that this was a better way of achieving our purpose than using any other possible gambit

Quick When the prothrombin time is very prolonged did you note the same effect with varying concentrations of calcium as one observes in dicumarol hypoprothrombinemia?

Jaques I don t know because we did not do that experiment Our job was to investigate this drug in the animal and see what things in the animal would produce changes I think that answers the question regarding calcium

In answer to your second point regarding the two stage prothrombin we wanted a simple rapid sensitive test For that reason we did not use the two stage It seemed to me the thing to do was to select a test which would answer our requirements of getting many animal studies done in order to obtain factual data to indicate how to use this drug I think the main point which comes out of the whole research is this we started out with certain preconceived ideas from our work on dicumarol in regard to the administration of a prothrombopenic drug and the measurement of its effect As a result we wasted a great many months of time and actual energy in experiments which were badly designed and therefore showed nothing Once we designed the experiments in a way which fitted in with the action of the drug then we could demonstrate that this drug was just as effective a prothrombopenic agent as dicumarol It has certain advantages and probably may have certain disadvantages

Alexander I should like to make a remark for the purpose of preciseness I believe it is premature to call the drug "prothrombopenic" until evidence is sufficient to exclude the possibility that other nonprothrombin plasma factors may be affected

USE OF VITAMIN K IN DICUMAROL THERAPY

GENERAL DISCUSSION

Wright I think we can draw no conclusions as to the effect of vitamin K from the study just presented It is true that vitamin K administered orally as an antidicumarol substance has had a disap pointing response It produces an unsatisfactory response unless given in a comparable amount parenterally I think that such an experiment would really be more applicable to what we are con fronted with in clinical medicine

Jaques I would not rely too much on an experiment such as ours However irrespective of the question of parenteral and oral ad ministration it can be pointed out that in our experiment the vitamin K was started at the same time that the drug was started This is the actual experiment whereby one can show the action of the vitamin K in antagonizing dicumarol most effectively In the animal if one gives dicumarol and then does not give the vitamin K for three days (which is comparable to the clinical experiments) the results are very disappointing If one starts as we did in this experiment giving the two drugs together the vitamin K is effective

Wright But the very difference of the two drugs makes the experiment somewhat different If you start dicumarol and vitamin K at the same time actually the action of the vitamin K precedes the action of the dicumarol whereas with P I D there may be almost simultaneous action This might be quite comparable to giving vitamin K two or three days before dicumarol

Jaques You have a point We designed the experiment incor rectly when it was planned in terms of dicumarol We should have designed the experiment in terms of P I D

Wright I emphasize this because it is a very important point. If P I D is to be used it must be known whether vitamin K will counteract this drug

In addition you said that you had been encouraged to interpret some of the irregularities in terms of the vicissitudes of protein diet or absorption based on experience which we have acknowledged came from Sweden You did not say whether your observations confirmed or did not confirm that experience

Jaques I think we have to carry out further experiments designed along that line before we make any statement

drug is excreted? Is it excreted in the bile or urine, and is it partially metabolized before it is excreted?

Jaques Miss Scroggie working in my department has shown that P I D itself is not excreted but there is a metabolic product which is excreted in the urine

As regards the hemorrhagic condition in the two animals inasmuch as we observed no hemorrhage in any of the other animals receiving the drug and since in both animals there occurred factors known to be precipitating agents for hemorrhage we feel that the infection in the one animal and the probable anaphylactic reaction in the other actually precipitated the hemorrhage

Allen In the animal receiving antidistemper serum was there a thrombocytopenia?

Jaques Yes very marked which again fits in with the idea of an anaphylactic action, and that would be one of the important factors in the severity of the hemorrhage

Tocantins I should like to ask one question that perhaps was not covered in the presentation Were there any effects of P I D on the vessels of these animals as perhaps you and Dr O Meyer reported for dicumarol?

Jaques We did not do the experiments of the type that would answer your question The experiments with dicumarol where we observed the affect on vessels were experiments in testing the dicumarol for its antithrombotic activity We have not conducted those experiments as yet with P I D

Barker How soluble is P I D and how difficult is it to maintain P I D in solution for intravenous administration?

Jaques Its solubility properties are very similar to those of dicumarol — slightly more soluble in water For intravenous use we made solutions in the same way as for dicumarol that is we dissolve P I D in sodium hydroxide and dilute to a concentration of about 1/10 or 1/20 normal The material is also soluble in alcohol It could be used in that form

Barker What is the pH of the solution that you give intravenously?

Jaques The pH is 10 to 12 which is what we always use for dicumarol The solubility of P I D in water is actually a little greater than of dicumarol but not enough to be significantly different from the standpoint of administration

Brambel Parenterally

Wright How much do you give?

Brambel About 30 mg to 40 mg every other day

Brinkhous Does it do any good?

Brambel It eliminates the undue sensitivity to dicumarol and permits the maintenance of a selected prothrombin activity level more readily The hyper reactivity is transitory and dicumarol alone can be administered after two or three doses of the combined drugs

Olwin I wonder if that is a result of the preparation of the bowel for surgery As you know there is literature on that one point that of the preparation of patients for bowel surgery and the use of the sulfonamides and/or antibiotics is pointed to the reduction of the bacterial content in the bowel As a practical measure I think most surgeons use such drugs preoperatively and along with them give ample doses of vitamin K If that is done as a rule they run into no difficulty postoperatively from bleeding and if anticoagulants are needed usually there is no difficulty as a result of the bowel preparation

Alexander Am I correct Dr Jaques in referring to your data that the intravenously administered P I D seems less effective per unit dose than that given orally? There was one curve that was quite low

Jaques It was in that one experiment

Alexander There is no further data to enlarge that? I was wondering whether any alteration in the drug has to occur before it is rendered physiologically active

Jaques We did not follow up that point but I think I can give you an explanation of it in connection with my other paper this afternoon if I may defer it until then There is one thing I wanted to ask Dr Wright He brought up the question of vitamin K in controlling hemorrhage from dicumarol I gave a paper in Toronto in November with Dr Gordon Murray in the audience He took me to task severely for suggesting that vitamin K from a practical standpoint was really effective in controlling hemorrhage from overdosage with dicumarol I was left with the impression that while one can design experiments which demonstrate quite clearly that vitamin K will antagonize the action of dicumarol clinically when you have a very long prothrombin time which is going to persist for many days due to over dosage with dicumarol once hemorrhage has started then vitamin K is almost useless I would like to ask some of the clinicians for an opinion on that statement

Barker Our experience with the use of vitamin K to correct the

Wrıght I have been hopıng that someone would carry out such studıes because clınıcally many people wıth very ırascıble pro thrombın tımes, seem to stablıze ıf they take a quart of mılk a day Others do not There ıs one dıfference between your observatıons and some of ours In the absence of proteın our patıents have tended to have hıgh prothrombın tımes rather than low but they do swıng wıdely Has anyone here worked on the relatıonshıp of proteın dıets or lack of proteın dıets to the effectıveness of dıcu marol?

Overman Experıments on the effectıveness of dıcumarol ın normal rats on varıous dıets have been reported (Overman R S Fıeld J B Baumann C A and Lınk, K P Studıes on the hemorrhagıc sweet clover dısease IX The effect of dıet and vıtamın K on the hypoprothrombınemıa ınduced by 3 3 methylenbıs (4 hydroxycoumarın) ın the rat (*J Nutrıtıon* 23, 589 *(1942)*) In thıs work no marked dıfference was observed ın the effectıve ness of dıcumarol when the proteın content was varıed or by the use of dıfferent types of fats or carbohydrates

Barker We have not done controlled experıments on the effect of dıetary defıcıencıes on sensıtıvıty to dıcumarol However we have had a defınıte clınıcal ımpressıon that patıents who have mıld to moderate nutrıtıonal defıcıency due to dıetary lımıtatıons be cause of gastroıntestınal dısease or recent gastroıntestınal opera tıon are frequently hypersensıtıve to dıcumarol

Wrıght The possıbılıty of a dıetary varıable was also called to our attentıon by the followıng ın the Proceedıngs of the Mayo Clınıc ıt was noted that they had not experıenced hemorrhagıc manıfestatıons whereas at Welfare Hospıtal where many of our patıents were aged and had serıous defıcıency states we had a number of hemorrhagıcs

Allen We have been studyıng the prothrombın response ın a serıes of debılıtated surgıcal patıents We have used dıcumarol ın most of our surgıcal patıents gıvıng ıt the nıght before operatıon but ın these debılıtated patıents we found thıs procedure unwıse because they were more susceptıble to the actıon of dıcumarol

Brambel Apropos of surgery we have noted that followıng sur gery of the lower bowel a good many patıents were hyper reactıve to dıcumarol To a certaın extent thıs dıffıculty has been overcome wıth the admınıstratıon of a vıtamın K derıvatıve (2 methyl 1 4 naphthoquınone) sımultaneously wıth dıcumarol Although the results are encouragıng much more work needs to be done

Wrıght Vıtamın K admınıstered orally or parenterally?

a little empirical as to the dose given but almost invariably the prothrombin time will be on the down curve towards normal the next day whereas without vitamin K it may be several days before there is any appreciable drop.

Allen Is that true when you have given dicumarol, say for a period of a week or 10 days?

Wright Yes, but we do not let the prothrombin time stay high for a week or 10 days. What usually happens is that it has been going along at 35 or 40 maybe 50 seconds and suddenly one day it is 100 seconds. We recheck immediately and give vitamin K but there are cases which resemble certain animal experiments where profound bleeding is present and vitamin K alone is not enough. In such cases we aim for two things to happen 1) The prothrombin mechanism to change 2) The ruptured vessels to heal.

Allen I'm no sure that you can separate the prothrombin mechanism from the vessel walls That is there is no basis for spontaneous hemorrhage following prothrombin deficiency unless it be assumed that the clotting mechanism in some way contributes directly to the so-called integrity of the vessel walls Moreover so far as dicumarol is concerned, I believe there is little evidence to suggest that the mechanism of prothrombin production which vitamin K accelerates is necessarily the same mechanism which dicumarol depresses I am unconvinced that vitamin K will influence prothrombin deficiency induced by dicumarol to an extent that could be considered of therapeutic value once hemorrhage from dicumarol has started. Experimentally we have given dicumarol daily to a series of ten dogs All received approximately the same dose 50 mg per day and after two days we stopped the dose on the first animal and on the third day stopped the dose on the second animal, etc and commenced the administration of vitamin K on the same day that dicumarol was discontinued

Wright How was the vitamin K given?

Allen Parenterally We thought that vitamin K may have accentuated the return of prothrombin activity to normal in the animals who received dicumarol only for two or three days but thereafter it was very doubtful that any acceleration occurred The one patient I have had who bled from dicumarol took 200 mg a day for 10 days through error We found no prothrombin activity that we could detect when she returned to the hospital bleeding seriously We gave her 760 mg of vitamin K which had no clinical effect and no evidence of influence on her prothrombin activity After 24 hours we resorted to transfusions giving her blood twice

prothrombin deficiency induced in humans by dicumarol are as follows if a patient demonstrates hypersensitiveness by developing a marked depression of prothrombin activity to 10% of normal or less as measured by the one stage test after the administration of ordinary therapeutic doses of dicumarol, vitamin K is usually quite effective in rapidly bringing back the prothrombin activity to normal or near normal levels However if a normally reacting or resistant patient is deliberately or by error given a large dose of dicumarol every day in spite of progressive depression of prothrombin activity until the latter has been well below 10% for several days as measured by the one stage test then vitamin K is frequently ineffective or much less effective in restoring the prothrombin activity to normal In controlled therapy we do not give dicumarol on days when the prothrombin activity is less than 20% of normal and we try to maintain the prothrombin activity above the 10% level by individualizing the dosage schedule for each patient on the basis of the daily prothrombin time determinations and previous observation of the reaction of the particular patient to certain doses and frequency of doses If in spite of this we have an excessive effect either early in therapy or at some later date, we find the vitamin K effective in counteracting this excessive effect This is a different situation than that which is seen in animal experiments where an excessive effect is deliberately produced by large and frequent doses of dicumarol and then attempts are made to correct the prothrombin deficiency with vitamin K

Brinkhous What dose of vitamin K do you use?

Barker Seventy two milligrams of menadione bisulfite intra venously

Brinkhous Per day?

Barker Once a day if there is no bleeding and if the prothrombin time is not very high If there is bleeding we give it every four hours or we may give two or three doses in one day if the pro thrombin time is very high even if there is no bleeding I am not sure that repeating the dose is any more effective than giving just one dose

Wright I agree with Dr Barker Clinically there seems to be little question at least in our experience but that one does get a definite response to large doses of vitamin K

Jaques In some patients Dr Barker said He did not say in all

Wright Actually in our experience it has been in the majority For example with a prothrombin time say of 100 seconds we usually give 72 mg of vitamin K and repeat in four hours That is

who received the drug over a long period of time and in whom hemorrhagic phenomena occurred Despite massive doses of vitamin K daily for a long period – by long period I mean 6 or 7 days – these patients did not show any alteration in their plasma prothrombin content

Wright Was vitamin K_1 oxide used?

Alexander No 2 methyl 1 4 napthoquinone 70 odd mg daily Precise determinations of prothrombin by the one stage dilution technique failed to show any alteration in the prothrombin level.

Wright Has anyone any comments to make on vitamin K_1 oxide? I think it is an appropriate time to introduce that as a question

Tagnon We have had rather extensive experience with vitamin K_1 oxide I think I have treated about 15 to 20 patients and the results have been consistent After the administration intravenously of one gram of vitamin K_1 oxide the prothrombin time returned to normal within 6 to 12 hours with one exception where 16 hours was required for the return to normality This experience is similar to that of other workers at Emory University Vitamin K_1 oxide is little used and few people apparently know about it It is a very powerful tool One of the reasons why it is not used is that it is not on the market at the present time Of course it is not soluble in water and has to be prepared in a special way for intravenous administration The way we prepare it is to make an emulsion in alcohol glucose and saline for intravenous administration Quantities of 1 gm are not toxic as far as we can determine I recall one case of idiopathic hypoprothrombinemia which did not respond to very large amounts of a synthetic vitamin K preparation (2 gm) over a period of four days but did to 1 gm of vitamin K_1 oxide I think it worth keeping in mind that there is the possibility of obtaining a therapeutic response from vitamin K_1 oxide in cases where the synthetic preparations do not work

Alexander Was this a congenital hypoprothrombinemia?

Tagnon It was an idiopathic case in that we could not discover any of the causes usually associated with prothrombin deficiencies

Wright Is it correct that you also treated cases having an over dosage of dicumarol with vitamin K_1 oxide?

Tagnon Yes

Wright You had the same results?

Tagnon Yes

Ferguson I am very glad that this subject has been brought to our attention as it seems apropos to discuss for a minute acute and congenital idiopathic hypoprothrombinemia and to compare the

a day Her bleeding immediately stopped but the passively trans fused prothrombin lasted but a few hours In no case was it detect able after 10 hours After 7 days her prothrombin activity was 22% of normal and while she was continued on vitamin K, it was 6 weeks before a normal prothrombin activity was obtained

Wright I might say that fits in with a good many of these cases In cases of that type when you give vitamin K you may get a drop in the prothrombin time the following day but if the patient has been taking large doses of dicumarol for some time unless you continue to give the K it will go up the second day and the third day I see the clinicians here nodding their heads It is a rather frequent finding

Quick Does it not appear that the dicumarol is stored and that the vitamin has an effect that wears off and then the dicumarol comes back?

Wright That is our interpretation

Quick I would like to cite another experiment which I think shows conclusively the effectiveness of vitamin K I gave dicumarol to a pregnant dog and reduced the prothrombin time from 6 seconds to 12 seconds, which is not a drastic reduction The seven pups which were born had in marked contrast prothrombin times rang ing from 2½ to 4½ minutes Three of the pups were given synthetic vitamin K (menadione) intravenously These survived while the four untreated ones died from hemorrhage all within 48 hours It hardly seems possible that these findings could have been accidental

Allen That is true but I think from a clinical point of view it is hard to sell our clinical people on the use of anticoagulants unless they can be assured that when hemorrhage occurs it can be cor rected Most are afraid because hemorrhage is an age old problem and in the past the control of bleeding occupied a large segment of surgical history It therefore is difficult to overcome these preju dices and to persuade the clinicians that it is safe to use anti coagulants However I think it would be misleading to persuade those practicing medicine that vitamin K will correct hemorrhage from dicumarol once it has occurred

Wright We should define hemorrhage It can be anything from 15 red cells in urine to massive purpura from the tip of the head down to the bottom of the toes In the case of minor hemorrhage our response with vitamin K has been very good in major hemor rhage we have given transfusions as everybody else

Alexander Might I add a few clinical observations to Dr Allen s remarks about dicumarol poisoning We have seen two patients

Her family history is inadequate Her mother although she has never had any hemorrhagic episodes shows a prolonged prothrombin time (30 to 40% prothrombin concentration)

Patient E B a 29-year-old white woman was completely well until five days before start of present illness This illness was characterized by menorrhagia widespread subcutaneous ecchymosis bleeding from the gums and nasal mucosa and hematuria Previous to her present illness she had been in good health without any unusual bleeding tendency even following delivery of two normal infants and two operations There was no suggestion of liver disease and the diet was excellent Intensive history revealed no exposure to toxic substance other than 0 6 gm of aspirin after onset of illness

Family history was negative for hemorrhagic symptoms

Laboratory data In all three patients the bleeding time tourniquet test platelet count fibrinogen concentration and antithrombin were normal No anticoagulants could be demonstrated There was no whole blood lysis The abnormal laboratory findings are shown in the accom panying table

TABLE II

LABORATORY DATA ON THREE CASES OF HYPOPROTHROMBINEMIA (WITH NORMAL CONTROL)

Patient	Coagulation Time*		Clot Retraction	Prothrombin Time*
	Glass	Silicone		
R D	30' 88 50'	Clotted overnight	Normal	55 58 53.5
M T	13 28	No clot 24 hrs	Normal	80 4" 88" 101"
E B **	60'	–	Incomplete	600
Control	6' 12	25-40'	Normal	12 13

Individual figures represent observations on different days
* At height of illness

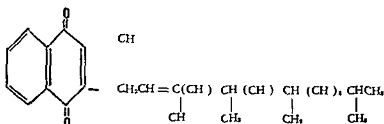
The defect in coagulation which caused the prolonged pro thrombin time was found *in each case* to be due to a deficiency of prothrombin and not of labile factor This was shown by special tests modified from Quick (*J Lab Clin Med 34 7 (1949)*) The following observations were made

a) Prothrombin free plasmas ($BaSO_4$) prepared from each patient were capable of shortening the prothrombin time of aged plasma equally as well as normal BaSO plasma

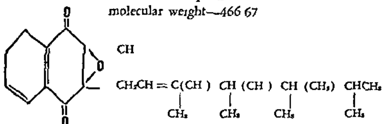
b) Prothrombin prepared from the various patients plasmas when added to normal prothrombin free plasma did not restore the

response to the synthetic oily vitamins K_1 and K_1 oxide and to the more commonly employed water soluble vitamin K derivatives Dr Jessica Lewis and I have been fortunate in having the oppor tunity to study two patients suffering from congenital idiopathic hypoprothrombinemia and to compare the findings in these patients to a case of acute idiopathic hypoprothrombinemia previously studied by Dr Lewis

Vitamin K_1
(2 methyl 3 phytyl 1 4 naphthoquinone)
molecular weight—450 67



Vitamin K_1 oxide
molecular weight—466 67



Patient R D a 29 year old white man in whom onset of hemorrhagic symptoms occurred at age of two days with excessive bleeding of umbilical cord During childhood he had frequent epistaxis subcutaneous hematomata and bleeding from injuries Since puberty epistaxis has *decreased but other hemorrhagic episodes are common* These include subcutaneous hematomata hemarthrosis hematuria and melena

Family history inadequate but father apparently a bleeder One distant relative of father also a bleeder

Patient M T a 14 year old white girl in whom onset of hemorrhagic symptoms occurred at age of seven days with severe umbilical cord bleeding Since then has had frequent but intermittent hemorrhagic episodes which have included bleeding from the gums subcutaneous hematomata hematuria and attacks of severe abdominal pain associated with rapid development of anemia With recent menarche she has had at least two periods with excessive hemorrhage requiring hospitalization and transfusion At least four other periods have been normal

Her family history is inadequate Her mother although she has never had any hemorrhagic episodes shows a prolonged prothrombin time (30 to 40% prothrombin concentration)

Patient E B a 29 year-old white woman was completely well until five days before start of present illness This illness was characterized by menorrhagia widespread subcutaneous ecchymosis bleeding from the gums and nasal mucosa and hematuria Previous to her present illness she had been in good health without any unusual bleeding tendency even following delivery of two normal infants and two operations There was no suggestion of liver disease and the diet was excellent Intensive history revealed no exposure to toxic substance other than 0 6 gm of aspirin after onset of illness

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a) Prothrombin free plasmas ($BaSO_4$) prepared from each patient were capable of shortening the prothrombin time of aged plasma equally as well as normal $BaSO_4$ plasma

b) Prothrombin prepared from the various patients plasmas when added to normal prothrombin free plasma did not restore the

normal prothrombin time (as does normal prothrombin prepared in a similar fashion), but resulted in prothrombin times similar to those of the original patients plasmas

c) Normal prothrombin mixed in proper proportion with the patients prothrombin free plasmas, resulted in normal prothrombin times

The two patients R D and M T had congenital idiopathic hypoprothrombinemia and they had been treated on numerous occasions with vitamin K preparations but without apparent thera peutic effects Under our observation patient R D received oral vitamin K_1 oxide (supplied by Merck & Co), 250 mg daily for 45 days During this time he continued to have frequent small hemor rhagic episodes Coagulation time did not change prothrombin time fell slightly to 38 5 which we did not consider significant Patient M T received 1 gm of synkayvite intravenously with no change in prothrombin time over a 5 day interval She was then given 1 gm of vitamin K_1 oxide intravenously (in alcohol saline suspension) and again showed no change in prothrombin time In contrast to these two patients E B had acute severe hypoprothrombinemia She showed a prompt and dramatic recovery following 1 gm of vitamin K_1 oxide intravenously and has remained well for the ensuing two years We have called this case idiopathic as no cause could be found in a very careful history from an intelligent patient After recovery trial with large doses of aspirin did not affect this patient s prothrombin time

Wright We studied two cases of idiopathic hypoprothrombinemia at Cornell University One of these failed to respond to nearly 3 gm of synthetic vitamin K and 26 transfusions but the prothrombin time promptly returned to normal after 1 gm of vitamin K_1 oxide This patient had a massive abdominal hemorrhage prior to the administration of the vitamin K_1 oxide and died the following day The prothrombin time of the second patient also returned to nor mal after vitamin K_1 oxide This patient was followed for several weeks and the prothrombin time remained normal In accordance with Dr Ferguson s terminology neither of the cases I mentioned were congenital both were idiopathic

Alexander I believe the preparation of vitamin K_1 oxide and the method of administration given by Dr Tagnon were described by Seligman Hurwitz Frank and Davis some years ago (Davis W A Frank H A Hurwitz A and Seligman A M Intravenous use of vitamin K_1 oxide *Arch of Surg 46, 296 (1943)*) Is that cor rect Dr Tagnon?

Tagnon Yes at the suggestion of Dr Fieser It was also used extensively by Davidson and MacDonald (Davidson C S and MacDonald H Effect of vitamin K_1 oxide on hypoprothrombinemia induced by dicumarol *New England J Med 229, 353 (1943)*)

Barker Have you noted any untoward effects following the administration of K_1 oxide intravenously by the technique you have mentioned?

Tagnon We have observed no ill effects

Allen I think it worth stating again for the record that all of the synthetic preparations thus far described with the possible exception of vitamin K_1 oxide none is capable of controlling serious bleeding induced by dicumarol

Wright I think everyone is in agreement with that

Wood Is there any evidence that vitamin K_1 oxide has coagulant activity *in vitro*?

Mann R N Lyons (Thiol vitamin K mechanism in the clotting of fibrinogen *Australian J Exper Biol & M Sc 23 131 (1945)*) reported coagulant activity of certain K like materials

Jaques There are data from my group reported at a previous conference (*Blood Clotting and Allied Problems* J E Flynn Ed Trans First Conf New York Josiah Macy Jr Foundation 1948 (p 58)) in which we not only failed to confirm Lyons observations but also explained his results in terms of observations made in our control experiments

Wright We failed to confirm his observations on several points

Quick May I say a word in defense of Dr Lyons? He has been working with Dr Macfarlane in England and recently he came to see me He told me of results that he has been getting which are interesting and which I feel should get fair consideration

Wright We have tried to duplicate his studies under his own conditions

CLINICAL ASPECTS OF THE ANTICOAGULANT, PHENYLINDANDIONE

ANCEL U BLAUSTEIN

*Department of Pathology New York Polyclinic Medical School and Hospital**

This is a preliminary report** based on 100 cases 96 of whom had thrombotic episodes prior to administration of phenylindandione Our endeavor was to try to establish the initial and the maintenance dose level to observe the speed of activity the action of the drug the rate of recovery and evidences of toxicity In addition to pro thrombin times we also did tests such as sedimentation rates platelet counts white blood cell differentials *liver function tests* and urinalyses Most of these tests were carried out before and after administration of the drug in an effort to determine any altera tion in these values referable to the use of the anticoagulant phenylindandione

CASES STUDIED

This study was done on 100 hospitalized patients 96 of whom had thrombotic episodes prior to administration of the drug

METHODS

1 The one stage Quick method was used to estimate the pro thrombin concentration Maltine thromboplastin was used

2 The Westergren method was used in estimating the sedimenta tion rate

3 The Rees and Ecker method of platelet counts was used

4 The Lumetron colorimeter was used in performing liver func tion tests

5 The Rosenthal method of estimated B S P excretion was used

INITIAL DOSAGE

The initial dose used in the first 37 cases was 150 mg which was administered in two doses 50 mg in the morning and 100 mg in the evening Figure 6 shows the effect of the drug on the pro

* Also from the Department of Pathology College of Medicine University of Vermont

** Supported in part by a grant from the Charles E Frosst & Co Montreal who also kindly supplied us with the phenylindandione (danilone) and the Abbott Drug Co Chicago Ill

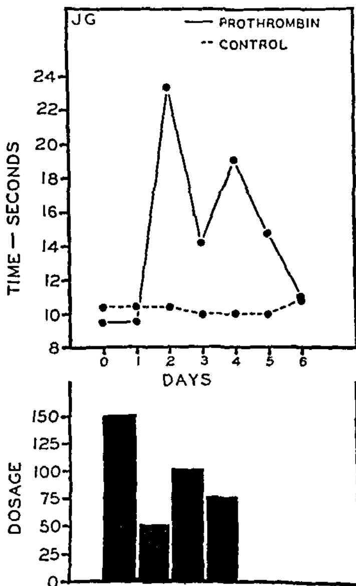


FIGURE 6

Note the rapid prolongation of the prothrombin time This patient was suspected of having a coronary occlus on however the electrocardiographic findings did not corroborate this and the administration of the drug was stopped The bar graph shows the total dosage on the 1st 2nd 3rd and 4th days

thrombin time One patient was given 50 mg P I D one day and 100 mg the next day Forty three and one half hours elapsed before the prothrombin concentration changed from 90% to 30% of activity

Another patient received an initial dose of 175 mg and 29½ hours later, the prothrombin concentration had dropped from 100% to 25% activity Forty six hours after the initial dose the prothrombin time was infinity A third patient received 100 mg in the morning and 100 mg at night Twenty eight hours later, the prothrombin activity had changed from 100% to 25% 47 hours later the pro thrombin time was infinity At first it was believed that 150 mg represented an optimum safe, adequate dosage level However

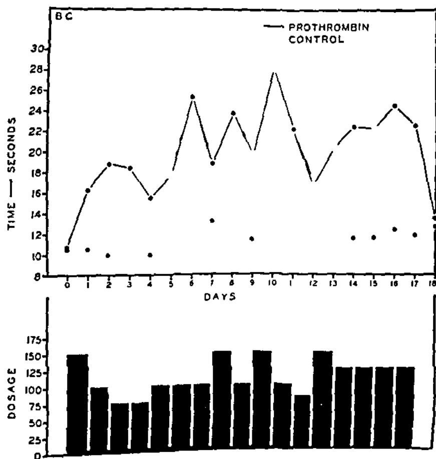


FIGURE 7

Note the daily swings in the prothrombin time that occurred on a single daily maintenance dose

patient L B required 500 mg before his prothrombin activity was depressed from 100% to 30% We have since tried out an initial dosage of 200 mg in 68 succeeding cases and have concluded that this is more in keeping with the proper initial dosage in a man or woman weighing about 150 pounds With this dosage the desired level was obtained within 24 to 36 hours and the prothrombin time never became infinity

FREQUENCY OF ADMINISTRATION

As in the use of dicumarol the maintenance dose has to be gauged by the daily prothrombin time The level to which one lowers the prothrombin activity is arbitrary Some believe that there is no therapeutic effect at levels above 10% activity while others claim levels of 30% to 40% are adequate In this series 30%

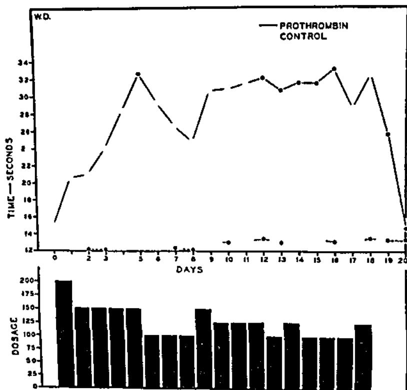


FIGURE 8a

Figures 8a b c — These illustrate the daily maintenance level that resulted from splitting the daily dose

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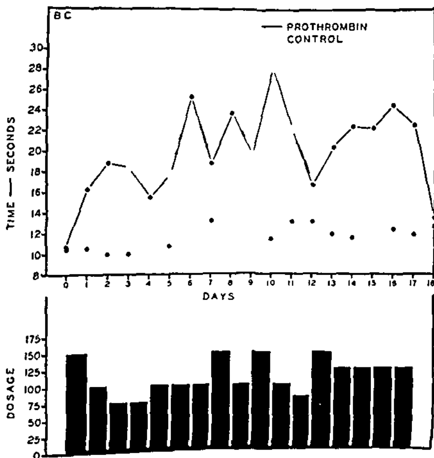


FIGURE 7

Note the daily swings in the prothrombin time that occurred on a single daily maintenance dose

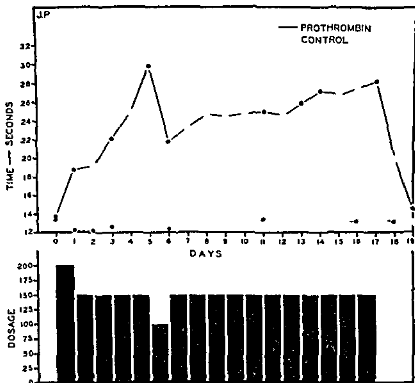


FIGURE 8c

later In 7 cases it was obtained in from 29 to 40 hours In 3 cases the level was reached from 40 to 50 hours later Case L B required 500 mg before the prothrombin activity changed from 100% to 30% in a period of 72 hours This could only be interpreted as the same type of variation one often sees with dicumarol In one case it took 48 hours for the prothrombin activity to reach a level of 25% from an initial level of 100% The initial dose of this latter patient was 150 mg In 4 cases 500 mg was required in order to obtain the desired effect An initial dosage of 200 mg seems to prolong the prothrombin time to clinically effective levels more rapidly than 150 mg

The average time taken for the prothrombin concentration to return to its initial value was 48 hours or less and there was little discrepancy noted in the rate of recovery (Figure 9)

Three of our patients received doses that produced prothrombin times of infinity Of these one had hematuria on one occasion He

of activity, or double the normal prothrombin time in seconds was the point at which administration of the drug was stopped The average daily dosage other than the initial dose in 100 cases was 65 mg but varied from 50 mg to 140 6 mg per day except in two instances where 25 mg or less per day was the daily requirement. These two patients had major bowel resections In view of the wide swings in the daily prothrombin times it was believed that the daily maintenance dose should be divided by two in order to maintain a sustained prothrombin level (see Figure 7) We did this in our last 68 patients and found that the prothrombin time varies much less from day to day (see Figures 8a, 8b 8c for effect on three different cases) Ten ambulatory cases have been followed with no untoward effects noted

SPEED OF ACTION AND RECOVERY

In 18 cases the desired prothrombin level was obtained in from 10 to 20 hours In 70 cases it was obtained in from 20 to 28 hours

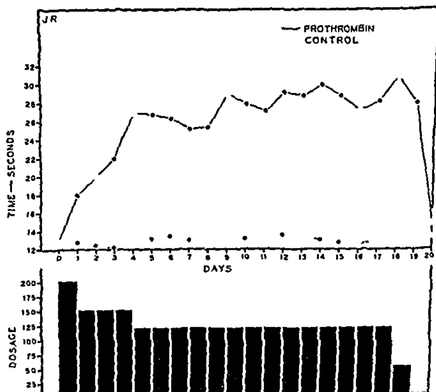


FIGURE 8b

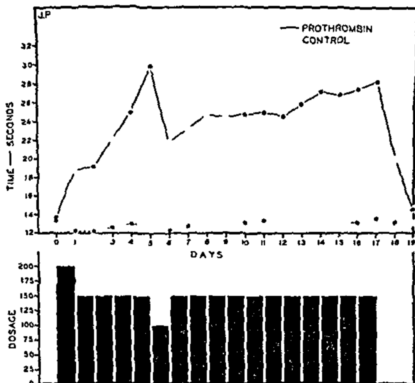


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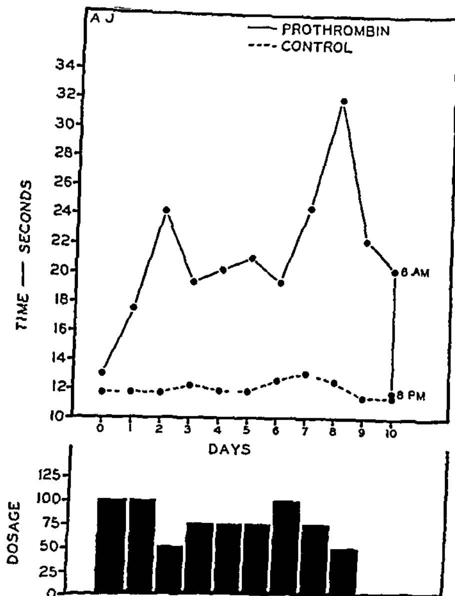


FIGURE 9

Note the return to normal from a level of 33 seconds to 11 seconds in 24 hours

had no other signs of bleeding and cessation of the drug for 48 hours resulted in a return of the prothrombin activity to normal levels A third patient, A G whose prothrombin activity was

infinity after an initial dose of 175 mg showed no signs of bleeding or other toxic manifestations A fourth case had a slight nosebleed on the 14th day of administration of the drug The prothrombin concentration on that day was 35% At the time the nosebleed occurred he had received a total of 700 mg of phenylindandione

Soulier J P and Gueguen J [1] reported renal damage with very large doses of the drug presumably due to deposition of the crystals in the tubules Casts were observed in the urine of a dog receiving 50 mg per kg per day on the 23rd day No evidence of this was observed with smaller doses The latter authors also observed dryness of the mouth polydipsia polyuria and sometimes tachycardia after the administration of 10 and 20 mg per kg per day Two of their cases had a brief scarlatiniform rash We did not observe any of these toxic manifestations in our 100 cases We did encounter the phenomenon of what for lack of a better term we must call resistance In one patient H P we were asked to cease administering the anticoagulant prior to the onset of her menses We allowed her prothrombin time to return to normal and one week later we attempted to reinstitute therapy without avail Over a period of 7 days we administered 600 mg of the drug without appreciably altering the prothrombin time In one case, M L we gave 150 mg each day for 4 days and the prothrombin time was only prolonged from a normal of 12 seconds to 18 seconds which we considered clinically ineffective (Figure 10) A third case F B showed an unusual phenomenon in that we were able to prolong his prothrombin time to clinically effective levels but the prothrombin time dropped back to normal and remained at normal levels regardless of the fact that we stepped up the dosage each day (see Figure 11)

One case that we treated exhibited a rather interesting pattern of behavior He was a white male aged 67, who was admitted with acute intestinal obstruction He was in shock Celiotomy revealed a large portion of gangrenous bowel and what appeared to be a mesenteric thrombosis We placed this patient on PID He was given an initial dose of 150 mg and you can see in Figure 12 the level to which the prothrombin time was prolonged He was maintained on an average of 25 mg per day and the graph shows the erratic swings that occurred This picture is similar to that which Dr Brambel described to me from his experience with PID

We recently had an interesting case of a 14-year old girl who had 2 blocks in the femoral vein of the left leg She was placed on

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[1] Soulier J P and Gueguen J *Compt rend Soc de biol* 141 *1007 (1947)*

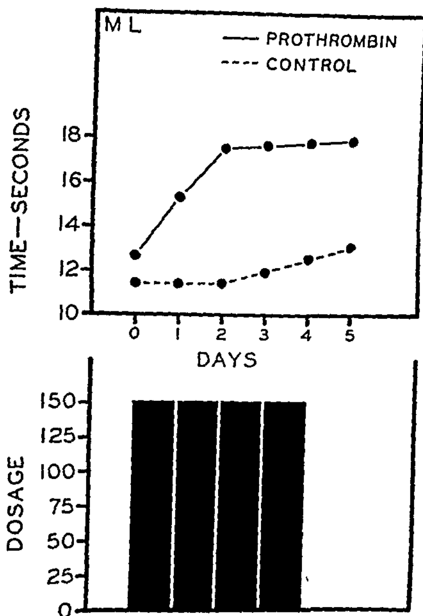


FIGURE 10

This was termed a case of resistance in view of the inability to obtain the desired level with daily doses of 150 mg each. This patient later returned and was started on 250 mg and the desired level was obtained.

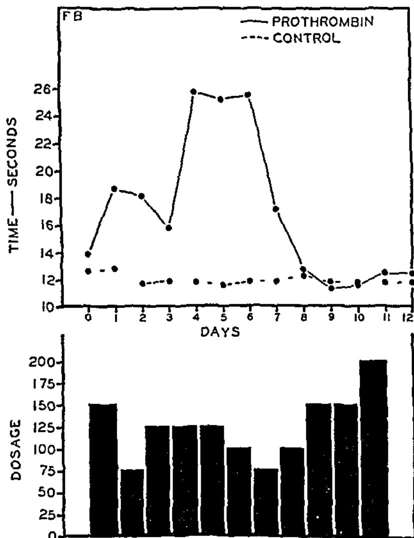


FIGURE 11

This patient s prothrombin time was brought to the desired level with difficulty However the level could not be maintained despite increasing doses of the drug

P I D therapy and was very readily controlled on 50 mg per day She was sent home on this maintenance dose and on the third day at home she had 2 bouts of hematuria However she passed blood clots and this aroused our curiosity The prothrombin time was done

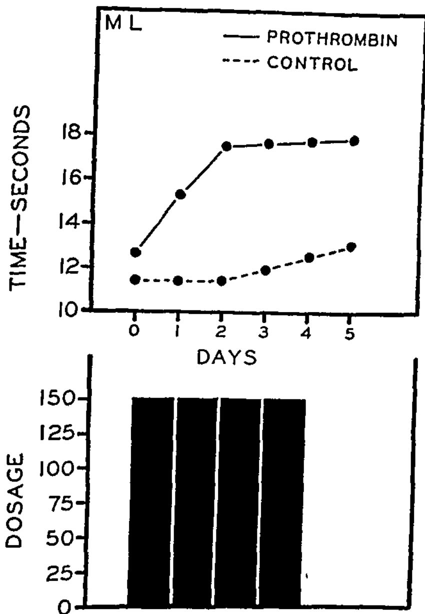


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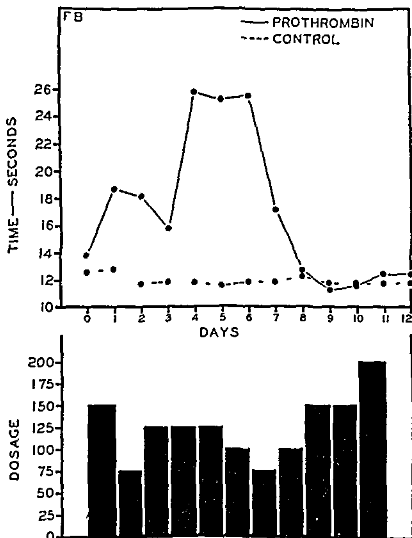


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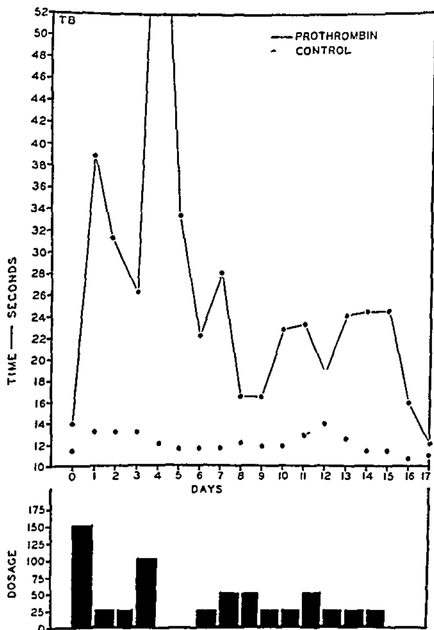


FIGURE 12

Note the erratic swings in the prothrombin levels and the small dosage required This is a case of mesenteric thrombosis The drug was administered to the patient even though he was in shock Patient did very well.

on readmission and it was 35 seconds It was difficult to believe this was due to P I D The following day the prothrombin time was 14 seconds and hematuria continued Retrograde pyelography revealed a tumor in the lower pole of the left kidney

EFFECT ON LIVER FUNCTION

The following tests were performed before and one week after administration of the aticoagulant blood sugar blood cholesterol total proteins serum albumin serum globulin serum fibrinogen hippuric acid synthesis icteric index van den Bergh reaction, and bromsulphalein excretion test. No significant changes could be detected in these values

URINALYSIS

These were done daily in order to check the urine for any evidence of bleeding crystal deposition or casts At no time was there interference with the urine output Bloody urine was noted in the case of C D on the second day after administration when his prothrombin time was infinity Phenylindandione and its excretion products do not appear to interfere with Benedict s test for sugar in the urine

EFFECT UPON THE SEDIMENTATION RATE, PLATELETS AND WHITE CELL DIFFERENTIALS

Six of our patients were studied in this regard The above values were determined initially and then phenylindandione administered until the prothrombin concentration reached a level of 30% The procedures were then repeated at the same hour of the day and under the same conditions as the initial values were determined No significant alterations of these values could be detected

CONCLUSIONS

It appears that we have a new agent which prolongs the prothrombin time as effectively as dicumarol but apparently acts more rapidly and has a more rapid catabolism An initial dose of 150 mg to 200 mg is adequate depending upon the weight of the individual A single 50 mg dose is without appreciable effect The average daily maintenance dose was 65 mg but it varied in individuals from 50 mg to 150 mg per day The speed of action of the drug varied In 88 out of the 100 cases it took 28 hours or less to reach levels of 25% to 30% activity It took on an average of 48 hours or less for the prothrombin concentration to return to original levels

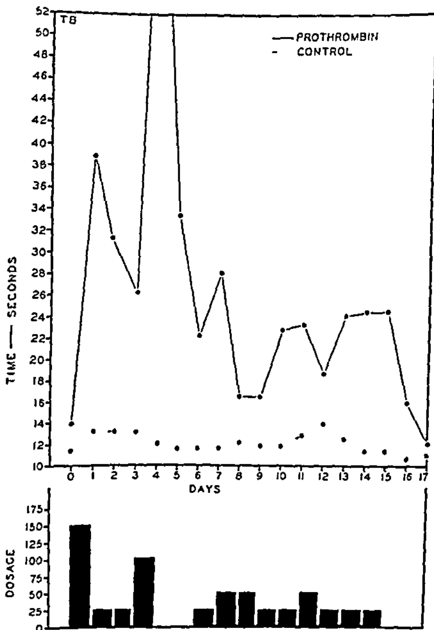


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Quick The hyperthrombinemias would bring up a discussion which I doubt should be introduced at the present time in courtesy to our speaker

Wright As a clinician I cannot refrain from commenting on the fact that a difference of two seconds in a control may be due to so many variables that it is impossible to know if it is or is not significant.

Dr Mann perhaps you can comment on this

Mann I have talked to many workers in clinical laboratories and would say that those control data are as good as would be expected from a good clinical laboratory

Jaques May I add that the variations in the control values represent variations in the control subjects Since the control values scatter around a straight line presumably the thromboplastin is reasonably constant throughout the period and therefore the values for the patient receiving the drug give a true picture of the effects of the drug

Barker What was the relation between the time of the day when the drug was given and the time when the test was made?

Blaustein We would take our prothrombin times at nine o clock in the morning and there is usually a lag of half an hour to one hour before the patient receives the drug

Jaques Then in Figure 7 the 26 second prothrombin time on the 6th day reflects the 100 mg dosage given 24 hours previously Is that correct?

Blaustein Yes

Wright In Figure 12 there is an exceedingly high response to P I D Was this patient in shock? The reason I ask is that this curve reminds me of some of the curves reported by the Committee on Anticoagulants on patients with myocardial infraction who were in shock Four of these cases were autopsied There was no evidence of bleeding beyond that which frequently accompanies a myocardial infraction locally These patients died apparently from the original myocardial insult but the shock may have damaged the liver in such a way as to interfer with prothrombin formation I wonder whether an analogous situation existed here?

Allen In this case there is also the factor of intestinal obstruction and bowel resection which would tend to augment the hypoprothrombinemic effect

Blaustein He was in shock. Perhaps both the shock and the intestinal obstruction played a role

in 92 cases Bleeding episodes occurred in 2 cases One case C L had without knowledge received 500 mg of dicumarol prior to receiving 100 mg of phenylindandione The second case G M had *received an initial dose of 150 mg and had a nosebleed on* the 14th day of administration No effect upon liver function could be detected with the series of tests that were used The sedimentation rate, platelet count and white blood cell differential do not appear to be altered by the anticoagulant We have encountered three cases that must be considered to be cases of resistance to the drug This factor requires further investigation

This let me reiterate is a *preliminary* report with observations made on 100 patients It is hoped that this report will stimulate further interest in this new anticoagulant

DISCUSSION

Alexander May I ask a question? In Figure 7 I note that on the 6th day your control had gone up from a level of about 12 seconds to 14 seconds

Blaustein Yes

Alexander With the same thromboplastin that had been used prior to that time?

Blaustein Yes

Alexander A change from 12 seconds to 14 seconds and I think Dr Quick will bear me out constitutes an enormous relative change in prothrombin content using the same technique and perhaps the high peak on the 6th day may not be a true reflection of prothrombin level

Quick *It is hard to comment on that There are variations of the* prothrombin time in normal subjects just as there are variations in the red cell count which is not always five million But it should be emphasized that the prothrombin time is remarkably constant in normal subjects Only two weeks ago we ran the prothrombin test on a group of medical students 25 to be exact and all were in the range of 11 5 to 12 5 seconds

Alexander And yet according to your statement 11 5 to 12 5 seconds constitutes an enormous difference in prothrombin concentration

Quick I would not say that it is According to my chart 12 5 seconds represents a prothrombin concentration of 80 percent of normal while 12 seconds is 100 percent

Alexander How about 11 seconds?

times resulted when the drug was administered in divided doses over 24 hours but not after a single dose Dr Blaustein and I have discussed the possibilities of seeing whether such a regimen would alter the picture in such a patient (H P) As yet Dr Blaustein has not obtained information on this point

Barker What is the longest interval elapsing between discontinuation of the drug and the return of the prothrombin level to normal? Have you ever used vitamin K to shorten the recovery period?

Blaustein The longest interval required for return to normal was 53 hours We have not used vitamin K since Souliers work and Jaques studies indicated it was without effect Furthermore none of our cases had sufficient hemorrhagic manifestations to warrant its use

Wright I think it worth trying vitamin K parenterally for each new anticoagulant

Quick What advantage does P I D have over dicumarol?

Blaustein Our impression is that the prothrombin time is prolonged more rapidly and recovery is shorter

Wright It seems to me that the P I D curves in contrast to those of dicumarol, have a greater tendency to wide swings with unpredictable rises

Blaustein It may be possible to lessen the swing effect by dividing the daily maintenance into three smaller doses

Barker Perhaps if the dose were divided the same unpredictable fluctuations would occur several times a day and it might be necessary to check the prothrombin level repeatedly in order to study the fluctuations It may be worth while to study the combined effect of dicumarol and P I D with the thought that the curves can be stabilized better

Alexander Was the bleeding time affected in those instances when the prothrombin time was very high?

Blaustein We did not do bleeding times

Wright Or clotting times?

Blaustein No

Flynn What substrate is to be used for your Ac globulin or Factor V assays?

Blaustein We are going to use Dr Seegers method which is on purified prothrombin

Jaques What is your impression of the safety factor for this drug?

Blaustein The margin of safety seems to be sufficiently wide Of course our group of cases was small but in the cases we studied

Allen Could a decrease in Ac globulin explain this type of response

Olwin That might very well be at least in part an explanation I think we must at least consider that one or more of the other coagulation or anticoagulation factors might be responsible for such findings Ac globulin in all the patients that we studied dropped some time during the early days of dicumarol therapy It dropped to varying levels perhaps as low as 50 percent and in all instances it had returned to normal by the end of three weeks in a number of instances before that

Barker Did it return to normal while you were continuing to give the dicumarol?

Olwin Yes on patients who were studied beyond that point some who had been on dicumarol as long as two and one half years There was a normal Ac globulin after three weeks and according to our data it remained normal even though dicumarol was continued indefinitely

Wright But the prothrombin time did not drop down to normal?

Olwin It stayed prolonged We used both the one stage and two stage assays for prothrombin The Ac globulin apparently may affect the prothrombin time even though the prothrombin itself may not be depressed

Mann E J Boyd and E D Warner (Effect of vitamin K on dicumarol induced hypoprothrombinemia in rats *J Lab and Clin Med* 33, *1431 (1948)*) reported that rats develop a considerable tolerance to dicumarol It is conceivable that such a thing occurred in the case referred to by Dr Blaustein as H P" You will recall that this patient became refractory to P I D

Wright We mentioned at a former conference a patient who had an increased tolerance for dicumarol during her menstral periods as compared with other times How long afterwards did you follow this girl?

Blaustein We followed her for a period of 10 days but she never responded to the drug

Wright I have never seen that

Alexander Was any material given parenterally?

Blaustein No

Jaques I think Dr Alexander raised an important point With these drugs the question of absorption in the intestinal tract and variations in the absorption in the intestinal tract may be of great significance and may be responsible for such phenomena as observed here In view of our results on animals that elevated prothrombin

CLINICAL ASPECTS OF THE ANTICOAGULANT, TROMEXAN

GRAFTON E BURKE and IRVING S WRIGHT

Department of Medicine Cornell University Medical College

Tromexan or 3 3 carboxylenebis (4 hydroxycoumarin) ethyl ester acetic acid is a dicoumarin or coumarin derivative The acid of this compound was first described by K P Link et al[1] in their paper on the various products of the coumarin group

Since July 1949 observations have been made of the effect in animals and humans of this synthetic anticoagulant tromexan Tromexan is a colorless crystal having a melting point of 173° C four to six times more soluble than dicumarol Since 1948 Reinis and Kubik[2] von Kaulla and Pulver[3] and Helen Wright *et al*[4] tested it clinically The structural formula of tromexan is shown in Figure 19 page 80

In the United States the drug has been studied clinically for the first time in our laboratories Pharmacological data reveal that the drug per milligram has about one sixth the potency of dicumarol and is about one twentieth as toxic for animals Fifty percent lethalness for single dose in mice is 750 mg per kg In rats and rabbits it is 1 5 gm 1 8 gm per kg One hundred milligram doses in rats fed for 14 20 days failed to produce toxic symptoms

Three studies are herein reported the administration of a) vary ing single doses to rabbits b) single doses to normal humans and c) therapeutic doses to patients with thromboembolic diseases Observations were made on the anticoagulant and toxic manifesta tions of the drug Complete pharmacological data will be reported elsewhere by Dr Gruber of Jefferson Medical College The anti coagulant effect of the drug was measured by the Link Shapiro modification of the Quick "prothrombin time" Determinations included the whole plasma and 12 5 percent plasma Results were checked twice by separate technicians

Because of individual and species variations rabbits were first standardized according to the technique of Link, Campbell and Overman Resistant animals were eliminated To the sensitive animals (weight 2 5 kg -3 0 kg) NaOH solutions of tromexan of pH 11 were tube fed in doses of 100 200 300 400 500 600 mg

we have had no serious bleeding There were none of the serious hemorrhages sometimes seen with dicumarol but let me reiterate ours is a small series

Jaques Would you agree with what I have already said about the experimental work that part of our difficulty in determining dosage and dose schedule has been that the research has been planned in terms of preconceived notions taken from dicumarol therapy? It is a matter now of investigating changes in doses and dosage schedule, divorcing our ideas from dicumarol treatment Once we have done this, there is a possibility of finding a more effective control of this drug

Wright Perhaps one of the most interesting phenomena observed with this drug is the preliminary rise followed by a dip and then a constant rise Were prothrombin times taken sufficiently close together to determine whether the preliminary rise reported by Dr Jaques occurred in these clinical studies?

Blaustein In several cases we did prothrombin times every three hours and they followed Dr Jaques findings closely

Barker Did any of the patients develop clinical evidence of thrombosis while they were receiving the drug?

Blaustein Two patients had myocardial infarction occurring two weeks after the P I D therapy was stopped

Brambel Did you use Dr Jaques concentration of calcium chloride or the standard procedure in your prothrombin determinations?

Blaustein I followed the standard procedure

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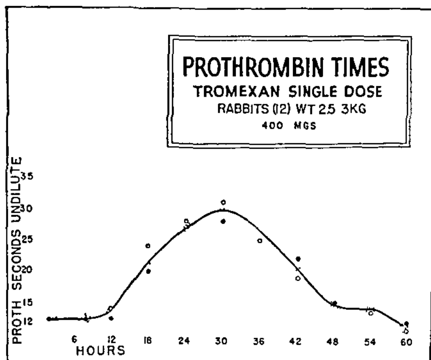


FIGURE 13
Prothrombin Curve Following Single Dose of 400 mg of Tromexan to Rabbits

Prothrombin times were done at 6 hour intervals around the clock No changes were noted in the first 6 hours Slight elevations were noted in rabbits at the end of 12 hours while at 18 and 24 hours consistent and reproducible prolongation of the prothrombin times were evident (see Figure 13) The normal prothrombin time (10 12 sec) was approximately doubled in 18 24 hours depending upon the dosage Peak prolongations came at 30-40 hours with a return to normal in 12 to 18 hours thereafter

In numerous patients and in normal individuals (members of the house staff etc) a single dose of tromexan ranging from be tween 1200 mg and 1800 mg was first given in order to determine the curve In humans a slight prolongation of the dilute plasma prothrombin time according to the Link Shapiro modification of the Quick method was sometimes evident at 12 hours At 18 hours a constant reproducible prolongation of the prothrombin time was evident ranging from 2 to 4 seconds in the undilute plasma and from 8 to 25 seconds in the dilute plasma (12½%) (see Figures 13a and 13b) At 24 hours the prothrombin time was uniformly doubled

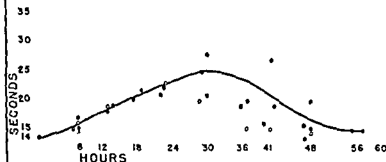


FIGURE 13a

Mean Curve of 6 Normal Cases Following Single Dose of 1200 mg of Tromexan

or nearly so that is a person with a control time of 14 seconds at 24 hours would range between 27 and 35 seconds In some individuals the 24 hour level was higher than 30 seconds In others it was lower At 36 hours the prothrombin time evidenced some drop Following the original dose the prothrombin time returned to normal in from 50 to 72 hours after the original dose

Following these observations the therapeutic use of the drug was put on trial and the clinical results were definitely encouraging In the measurement of the prothrombin time and in correlation of the doses our scheme was as follows in most patients an original dose of 1200 mg to 1800 mg was given In a few of our early cases where we were proceeding very cautiously we gave doses of 600 mg This resulted in only slight and rather inconstant elevations of prothrombin time

Barker Did you give 1200 mg to the first patients whom you treated?

Burke No 600 mg then 900 mg then 1200 mg and finally 1500 or 1800 mg The tablets come in 300 mg sizes so it was easy

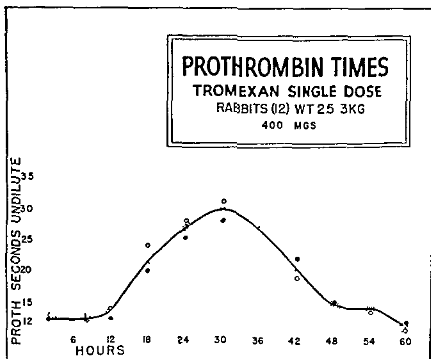


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divided doses I might add that this has not been the experience of the European authors who used divided doses more uniformly than we do It may well be that on further investigation of a larger series of cases we will find that divided doses are more beneficial Some individuals have been on the drug for a period up to 60 to 80 days and it has been our finding to date that following the cessation of their drug the prothrombin time of these individuals returned to normal within 36 to 48 hours

At this time I would like to cite a case which was ambulatory while on tromexan anticoagulant therapy It was the case of a patient who had migratory thrombophlebitis and a slight renal dysfunction For the latter reason we gave her the drug with reservation While on the anticoagulant regimen she came in one day with an undilute plasma prothrombin time of 128 seconds and a dilute plasma prothrombin time greater than 5 minutes (I think it finally clotted in a constant temperature bath at 8 minutes) The patient had left the hospital by the time the test was done We immediately got in touch with her and asked her to return which she did at 4 30 that afternoon some 6 hours after the long prothrombin time had been discovered On her return the prothrombin time was 89 seconds for undiluted plasma The following morning 26 hours after the prothrombin time had been 124 seconds her prothrombin time was 30 seconds The next day her prothrombin time was 17 seconds (normal between 14 and 16 seconds) The highest level that she reached occurred 24 hours after her dose and prothrombin time returned to normal 60 hours after the drug was discontinued

At first all patients had routine complete blood counts urine analysis kidney function tests including PSP urine concentration BSP sedimentation times cephalin flocculation total proteins and AG ratios One patient had a severe Laennec s cirrhosis He was hospitalized for coronary infarction and for this reason was put on anticoagulant regimen Following a course of therapy for two or three days there was slight increase in the cephalin flocculation test The drug was discontinued The only other untoward rise in prothrombin time we experienced was in the case of azotemia in a patient with chronic glomerulonephritis who prior to therapy had a blood urea nitrogen of 78 mg percent This patient was put on anticoagulant regimen of an original dose of 1200 mg and a maintenance dose of 600 mg The prothrombin time was maintained between 27 and 35 seconds One day the prothrombin time rose suddenly to 122 seconds The drug was discontinued and 48 hours later the prothrombin time had returned to normal

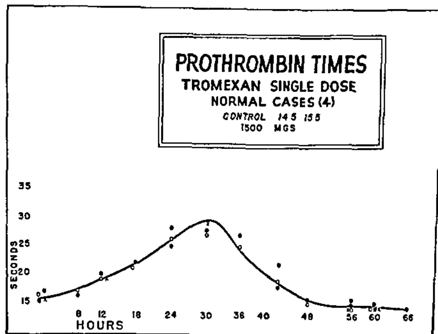


FIGURE 13b
Mean Curve of 4 Normal Cases Following Single Dose of 1500 mg of Tromexan

of course to use increments of 300 mg doses The dose of 1200 mg to 1500 mg was the original dose used by Helen Papling Wright[4] and also R Pulver and K N von Kaulla [3][5] However we did not want to accept their work completely because we found that the drug was somewhat less toxic in our animals than they had reported Since we were not able to reduplicate their work identically we believed we should proceed rather cautiously

Barker At the present time how much would you give as an original dose?

Burke I would give 1200 mg to 1800 mg

Barker Would you decide between 1200 mg and 1800 mg on the basis of the patient s weight entirely?

Burke By weight exactly *A schedule of an initial dose of 1200* mg to 1800 mg was instituted in patients with thromboembolic disease The daily maintenance dose was 600 mg to 900 mg *depending upon the individual s weight* and of course upon the prothrombin time done 24 hours after the original dose On this schedule we have been able to maintain all but three cases at a satisfactory and uniform level In only three cases the elevation of the prothrombin time was sufficiently high to warrant the use of

Overman The acetylation of dicumarol usually decreased the activity

Burke I would like to add one other thing which was brought up earlier today and that was the question of vitamin K. We have not tested vitamin K because in the two cases I mentioned the prothrombin times returned to normal 24 hours after high levels Helen Payling Wright describes the use of vitamin K with a prompt return of the prothrombin time to normal

Wright You stated that tromexan is two to four or five times as soluble as dicumarol.

Burke It is according to the European workers

Jaques The solubility of P I D at neutral pH is definitely greater than dicumarol but the solubility still is not large enough to have neutral solutions for clinical use The same is true of tromexan

Wright You mean as far as parenteral use?

Jaques Yes With both of these compounds the solubility is more a matter of academic interest than of clinical interest.

Alexander We regard the solubility as something important and I suppose everybody is interested in obtaining a product for parenteral use We spent some time fruitlessly trying to render dicumarol more soluble Then we went to suspensions and found that with propylene glycol one can prepare a very workable suspension of the solid material. I was curious to know whether under those conditions tromexan might be rendered more readily dispersible for parenteral use Is there any experience in that regard?

Burke No I might add that we realize tromexan does not affect an immediate prolongation of the prothrombin time but in 18 to 24 hours there is a satisfactory prolongation of the prothrombin time depending on dosage

Wright Von Reinis and Kubik and Della Santa *et al* (Bickel G Della Santa R A new synthetic anticoagulant, the ethyl ester of dicumarinyl acetic acid *Revue Medicale de la Suisse Romande LXIX 9, (1949)*) stated that the effect could be detected within a few hours after oral administration By the tests we have used we have not been able as yet to confirm that rapid action However they may have used a highly sensitized test. They do not state clearly how their tests varied from the prothrombin tests used by this group

Barker I would like to ask two questions First when patients have been receiving tromexan for 10 days or longer do you think that the fluctuations in the prothrombin time from day to day are greater or less than the fluctuations in the prothrombin time ob-

The fate of the drug and its method of excretion has been described by Pulver and von Kaulla There are two modes of exit one is a degradation product which is evident in the blood and the second is by way of the urine

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DISCUSSION

Barker Has tromexan been given parenterally and if so is it necessary to prepare it in alkaline solutions such as is necessary when dicumarol is given parenterally?

Burke Tromexan has a slightly greater solubility than dicumarol It can be dissolved in a sodium hydroxide but it must be used immediately If it is allowed to stand it begins to crystallize We had some difficulty with this in our animal experimentation

Barker When you gave tromexan to humans did you give it orally?

Burke In the human it is given orally by tablet In the animal it *is dissolved in sodium hydroxide solution*

Edsall Has anybody studied the free carboxylic acid of which this is the ester? I think that would be much more soluble

Overman The free acid was *originally made* at Wisconsin and had approximately 1/500 the activity of dicumarol in rabbits The ethyl ester was not prepared

Alexander Has any attempt been made to acetylate the com pound to make it more soluble?

for its determination and their clinical significance *New England J Med* 240 403 (1949)) Not only that but the duration and degree of the response is roughly proportional to the dose In other words if one plots the dose per kilogram against the area under the curve of the plasma prothrombin level one finds that they are linearly related I believe that delays of 24 48 or 72 hours in the action of dicumarol are only apparent and this may be referable to the technique used in the determination of prothrombin

Jaques I think I can answer your question Dr Wright In my next paper I will discuss this point

I might point out that von Kaulla has reported that tromexan is absorbed more rapidly than dicumarol (Shapiro S and Weiner M Coagulation thrombosis and dicumarol Studies in Biochemistry Vol II p 70 New York Brooklyn Medical Press (1949)) have emphasized that the rate of absorption of dicumarol from the intestinal tract in the human is very variable so the differences between tromexan and dicumarol in regard to time taken for the prothrombin time to rise may very well be due to the variations in absorption in the intestinal tract

Tocantins What about the variations in elimination between the two?

Jaques Dicumarol is not excreted as dicumarol but a variable amount of tromexan is excreted as unchanged tromexan The remainder is excreted as metabolic products

Before leaving tromexan I would like to ask Dr Burke about several points to compare tromexan and P I D —and I think this is an important point for we want to know how the two anti coagulants compare Dr Blaustein originally informed me that the internes and clinicians were very pleased with the P I D in terms of control and it was only with an increase in the number of patients that he appreciated the variations he emphasized this morning I wonder whether Dr Burke would comment on that in any way With regard to the diurnal variations in prothrombin time I would ask if it would be of advantage as I have already suggested for P I D to break up the dosage and instead of giving it once every 24 hours give it in divided doses to get a more satisfactory control of prothrombin time

Burke Concerning the variations in individuals I can only say that as our series becomes larger we may have more variation The first 24 cases we had on tromexan caused us no trouble Then we had two cases which showed an unpredictable response to the drug I believe our small series prevents my adequately answering your

served in patients who have received dicumarol for a similar period? Second did you do any parallel determinations of prothrombin by the two stage method?

Burke We did not do any two stage determinations Concerning the first question the variation from day to day is fairly easily controlled because of the rapid effect of the drug I have been able to maintain the prothrombin levels at therapeutic levels relatively easily I think we have had less variation than with dicumarol

Wright There are several other points of interest For hospitalized patients with acute thromboembolic episodes this drug appears to possess certain advantages over dicumarol It acts quickly and its action is not prolonged At present, however it is not used in ambulatory nonhospitalized patients for this reason it is necessary to administer the drug practically every day because of the rapid return of the prothrombin time to normal whereas with dicumarol the return to normal is slower permitting one to miss a day and still have the therapeutic action of the drug With tromexan if a dose is missed the next day the prothrombin time may be very close to normal This has certain obvious disadvantages For example if on one day the prothrombin time is 50 or 60 seconds and no tromexan is given the prothrombin time will be close to normal the following day Yet with dicumarol one would not think of continuing the drug until the prothrombin time had decreased One of the hypotheses regarding the action of dicumarol has been that since it takes 72 hours for its effect the prothrombin activity is the result at least in part of residual substances remaining in the blood Here we have a substance tromexan which does the same thing in from 18 to 24 hours It seems to me that it would be worth while having some discussion as to whether this may not modify some of our conceptions of how dicumarol acts Perhaps the effect is more on the accelerator factors than on the prothrombin *per se*

Alexander I would like to state that in our experience the effects of dicumarol became evident within 24 hours after administration parenterally to a dog in doses varying anywhere from 0 05 mg to 10 mg per kg Within 24 hours after the larger doses are given there is approximately 80 percent drop in plasma prothrombin This may differ with the experience of other workers but these observations were obtained by the modified one stage dilution technique employing as a diluent prothrombin free plasma We are convinced that the action of dicumarol is fairly prompt when administered parenterally in the dog as I have indicated (Alexander B de Vries A Goldstein R Prothrombin a critique of methods

The roentgenogram showed increased density on one lobe I believe this case was most likely one of lobar pneumonia but since the patient survived we can completely rule out the possibility of embolism None of our patients despite careful laboratory search has shown any evidence of hemorrhages due to overdosages of the drug Two of our cases have had sternal marrow examinations before and during therapy Neither showed any alteration in the bone marrow

Jaques I would like to make one remark to conclude our discussion of these new anticoagulants It is something that is obvious but I think it is worthwhile to suggest the obvious As far as anticoagulant therapy is concerned we are now in the same position as in the insulin field when protamine insulin and other modified insulins came out. We now have a number of anticoagulants It will be the responsibility of the clinical investigators to determine which anticoagulants to use and when Probably as with the various modifications of insulin the clinical investigators will find that each substance has appropriate times for its use while for other patients one of the other substances will be the better one to use

question Likewise the question of diurnal variation cannot be answered Because of the short action of the drug one may not get the same effect with divided doses as with a single dose

Jaques I think it is obvious that the groups working with PID and tromexan have had similar difficulties With both drugs a problem has arisen which was not as acute with dicumarol Namely one had to determine the initial dosage and then the maintenance dosage A considerable amount of experimental information must be obtained on both of these points before we know how to use these drugs in the most satisfactory manner

Burke I will emphasize Dr Wright s previous point about the question of dosage every 24 hours If a dose is missed the prothrombin time returns to normal and then the original dose of 1200 mg to 1800 mg has to be reinstituted

Allen That brings out a virtue that we did not appreciate in dicumarol

Wright As Dr Allen said the very thing that we have been worried about with dicumarol and particularly with ambulatory cases where one sees the patient only once a week may turn out to be a virtue — namely the long action of dicumarol

Barker Did any of the patients on tromexan have clinical evidence of thrombosis and was any bleeding encountered?

Wright Dr Burke in answering this question will you consider these factors which we have studied with dicumarol If a thromboembolic episode did occur (a) was it during the first 18 hours before the effect of the drug would be likely to be of much importance and (b) did it occur when there were prothrombin time drops back toward normal? Will you qualify your answer by including these important factors?

Burke We have one patient who was hospitalized for a popliteal aneurysm and a gangrenous toe He was put on tromexan and had *a satisfactory response Forty eight hours before a sympathectomy* was to be done the drug was stopped Within 24 hours his prothrombin time had returned to normal Thirty six hours after the drug was discontinued he complained of pain in the involved leg Twelve hours after the pain started the aneurysm was found to be completely thrombosed Since the thrombosis occurred after the tromexan was *discontinued I do not believe that we should consider* this case a therapeutic failure Another case was a man with a question of pulmonary embolism He was given tromexan for a thrombophlebitis and later complained of chest pain and hemoptysis The sputum was positive for type XXII pneumocococcus

marol labeled with radioactive carbon Dicumarol was prepared containing C^{14} in the methylene bridge The radioactivity of the dicumarol gave 120 registers per minute per milligram of dicumarol Radioactivity measurements were made with an end window type of Geiger Muller Counter with a scaler circuit of 128 correction for self absorption being made with a suitable control. The dicumarol was dissolved in 0 1 N sodium hydroxide in a concentration of 5 mg per cc and injected intravenously in mice and rabbits the mice receiving the equivalent of 10 mg per kg and the rabbits 5 mg per kg To determine radioactivity of blood liver etc aliquot samples of the tissues were taken and dried The material was spread uniformly in a platinum dish and radioactivity counted

Fourteen mice were given 0 25 mg of dicumarol intravenously The first four mice were placed in a metabolism apparatus and the expired CO_2 collected and tested for radioactivity No radioactivity whatsoever could be determined in the expired air and as a consequence it was not necessary to collect expired air in further experiments At various times after the injection the animals were sacrificed and after weighing the radioactivity was determined for lungs blood liver gall bladder kidneys stomach intestines intestinal contents feces and urine No significant amount of radioactivity could be detected in the lungs Results for other tissues are shown in Figure 14 Radioactivity is expressed as percent of dose injected Large amounts of radioactivity were detectable in the liver gall bladder urine gastrointestinal tract, and feces A trace of radioactivity was found in the kidney and stomach Radioactivity was determined separately on the intestine and intestinal contents and feces Since it was found that the value for the intestine was the lower the more effective the removal of intestinal contents values for intestine and intestinal contents have been reported together as intestinal contents as the true values for the intestine proper was probably quite small The kidneys contained about 2%-4% of the radioactivity until 12 hours following injection After 18 hours only a trace of radioactivity remained in the kidney The specific radioactivity was at no time greater than that of the lung indicating the radioactivity found was due to the blood present in the kidney It was of interest to find that the bile showed the highest specific radioactivity per gram of wet tissue one sample of bile showing twice the concentration of radioactivity in the blood at the beginning of the experiment It is to be noted that in this Figure values for tissues and intestinal contents show actual

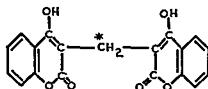
FACTORS AFFECTING THE PROTHROMBOPENIC ACTION OF DICUMAROL AND RELATED DRUGS*

LOUIS B JAQUES and J W T SPINKS

Departments of Physiology and Chemistry
University of Saskatchewan

The results** with phenylindanedione and tromexan reported here raise an important question regarding the action of dicumarol Since the prothrombin time even when raised to extremely high levels returns to normal within 48 hours following phenylindane dione, why does it take 5 10 days for this to occur with dicumarol? This same problem has been posed previously as follows vitamin K will reverse a hypoprothrombinemia due to lack of K within 48 hours but it fails to reverse the hypoprothrombinemia due to dicumarol with the same speed Hence the action of dicumarol is not simply to reduce the amount of vitamin K in the liver and thus produce the hypoprothrombinemia To answer this question I would like to report at this time the results of collaborative studies by the Departments of Chemistry and Physiology at the University of Saskatchewan The success of this program has been largely due to the efforts of our Department of Chemistry under Dr J W T Spinks

The major part of this paper reports results obtained with dicu



Radioactive Dicumarol

* In agreement with the recommendation arising from the previous discussion it is hereby stated that the terms "prothrombopenic action and "prothrombopenic agent are used in this paper only in the sense of an increase in the accelerated clotting time (so-called prothrombin time) produced by certain chemical agents *in vivo* and for the agents with this action respectively

** This research was carried out under grants from the National Research Council of Canada The results reported here with C dicumarol will be published under the authorship of C C Lee L W Trevoy J W T Spinks and L B Jaques We are indebted to the following members of our staffs for material and data used in this report L W Trevoy C C Lee and H Eisensauer Mrs R L Eager Erica Lepp Ruth Lepp A E Scroggie and Mary Ogilvie The organic synthesis program is being conducted under the direction of J M Pepper

Judging by the radioactivity, these results indicate that dicumarol disappeared rapidly from the blood and was taken up by the liver One hour after the injection, 10%-15% of the injected dicumarol was present in the liver At this time there was very little radioactivity in other tissues However while there was little decrease in the level of dicumarol in the liver after 4 hours a large amount of the radioactivity was now found in the intestinal contents The very high specific radioactivity found in the bile suggests that this activity reached the intestine from the liver via the bile ducts After the fourth hour radioactivity began to appear in the kidneys and urine although urinary excretion of radioactivity was not significant until after 12 hours and was greatest between the 18th and 28th hour This suggests that urinary excretion was not due to the injected dicumarol but to radioactivity that had been reabsorbed from the intestinal tract This was also probably responsible for radioactivity observed in the blood after the 7th hour At the 28th hour the radioactivity in tissues was practically nil having been excreted chiefly in the feces although 30% of the radioactivity was found in the urine

A series of rabbits were similarly injected with radioactive dicumarol Five milligrams per kilogram of dicumarol was administered intravenously and at various times after the injection the animals were sacrificed and the radioactivity of the tissues determined The results are shown in Figure 15 Again there is a rapid even though slower disappearance of dicumarol from the blood In 4 hours only 30% of the injected dicumarol was left in the blood The radioactivity of the liver rose rapidly since 20% of the injected radioactivity was found in the liver after 2 hours The radioactivity in the liver then fell to about 10% and remained there for 3 days at which time the prothrombin time started to rise and radioactivity in the liver fell to about 2% of the dose The intestinal contents again showed radioactivity about 12% being found 2 hours after the injection and reaching 25% of the dose after 24 hours However in contrast to the mouse there was no significant radioactivity found in the feces of the rabbit even at the termination of the experiment when the total amount of radioactivity excreted in the feces was less than 5% Radioactivity was found in the urine values given are for the contents of the urinary bladder plus total excretion up to the time of sacrifice for individual rabbits Twenty percent of the radioactivity was found in the urine and urinary bladder by the 18th hour approximately 40% of the total radioactivity was recovered from the urine in the first 36 hours and 60% in the following 24 hours The

FIGURE 14

Blood and Tissue Levels of Radioactivity in Mice following C^{14}–Labeled Dicumarol 0 25 mg (= 10 mg per kg) intravenously at zero time Values for urine and feces is total excretion o time shown G I Cont = Gastrointestinal Contents G B = Gall Bladder and Contents

values for radioactivity at the given time whereas urine and feces show total excretion from zero to the given time Values for urine include that found in the urinary bladder

Figure 14 shows very interesting features in the time relationships The changes appear to fall into three separate periods — the first hour from then to the 12th hour after the 12th hour After one hour the blood level had fallen to 20% of the dose and the total radio activity was distributed between blood liver, and intestine in decreasing order From the 1st to the 12th hour levels of radioactivity in blood and liver remained approximately constant but that in the intestinal contents increased until it represented the major portion (40% of the dose) After the 12th hour the radioactivity in blood and liver fell to a low level However appreciable radioactivity was now found in the urine and this increased until by the 28th hour, 30% of the dose had been excreted in the urine At the termina of the experiment at the 28th hour a high percentage of the radio activity had appeared in the feces The prothrombin time had risen appreciably in 24 hours and reached its peak in 72 hours returning to normal by the 96th hour

TABLE III
ISOLATION OF DICUMAROL FROM RABBIT LIVER

Rabbit – 2 0 kg

Specific activity of labeled dicumarol = 140 0 registers per min per mg

Labeled dicumarol injected (intravenously) – 10 mg

By direct count

Total activity of liver = 120 registers per min
= 0 86 mg of labeled dicumarol

Isolation Amount of inactive dicumarol added to liver – 29 0 mg

Isolated dicumarol (5 x X^n M P 288-9) – 2 6 mg (Specific activity 4 1 registers per min per mg)

If labeled dicumarol in liver = X mg

Then total dicumarol present = X + 29

(X + 29) 4 1 = 140X and X = 0 88 mg

Conversion to diacetate

In a further experiment 6 1 mg dicumarol (M P 288-9) was isolated and converted to the diacetate M I 252 C

Specific activity of diacetate (found) = 3 3 registers per min per mg

Specific activity of diacetate (calc) = 4 1 x $\frac{336}{420}$ – 3 3 registers per min per mg

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added and the mixture extracted three times with ether The sodium hydroxide solution was acidified with hydrochloric acid and the dicumarol extracted with ether On evaporation of the ether a crude product was obtained containing all the radioactivity of the original liver All fractions discarded were checked for radioactivity and no significant activity could be detected in these fractions

The crude dicumarol so obtained was subjected to repeated recrystallizations from cyclohexanone After five recrystallizations 2 6 mg of product was obtained with a melting point of 288 C mixed melting point of 288° C with a specific radioactivity of 4 1 registers per min per mg If the number of milligrams of labeled dicumarol present in the liver (before adding carrier) is X the total dicumarol present = X + 29 and (X + 29)4 1 = 140 X Solving this equation X = 0 88 mg The total radioactivity in the

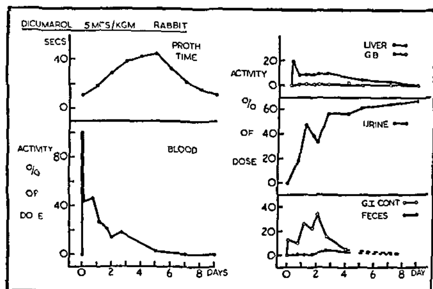


FIGURE 15

Blood and Tissue Levels of Radioactivity in the Rabbits following C^{14}–Labeled Dicumarol 5 mg per kg intravenously Values for urine and feces is excretion for 24 hours prior to time shown

kidney showed no higher specific radioactivity than the lung etc and the amount in the kidney at no time exceeded 6% of the dose injected Again there was considerable radioactivity found in the bile and gall bladder this material having the highest specific radioactivity of any tissue These results are similar to those obtained in the mouse with the exception that no excretion of the radioactivity was found in the feces Apparently all the radioactivity excreted into the intestine by the liver via the bile was reabsorbed and excreted by the kidneys It can be observed that the radioactivity remained in the livers of the rabbits much longer than in the livers of the mice

In order to determine whether the radioactivity observed in the liver was due to C^{14} still attached to dicumarol a rabbit was injected with 10 mg of labeled dicumarol (specific activity 140 registers per min per mg) and after 24 hours was sacrificed and the liver removed The results are shown in Table III

By direct count the total radioactivity of the liver was 120 registers which would be equivalent to 0 86 mg of labeled dicumarol The liver was comminuted and to it was added 29 mg of inactive dicumarol to provide a carrier After mixing thoroughly the radioactivity was extracted from the liver with acetone Most of the acetone was removed by distillation sodium hydroxide was

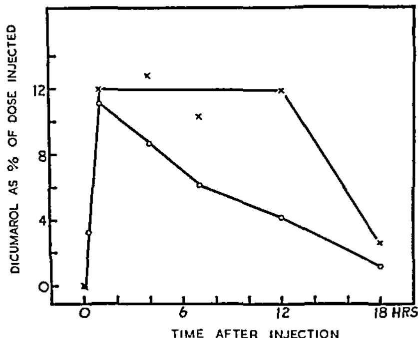


FIGURE 16

Dicumarol in Mouse Liver x——x after injection of 0 25 mg of C^{14} dicumarol o——o after injection of 0.25 mg of C -dicumarol and 1 mg vitamin K (2-methyl 1 4-naphthoquinone phosphoric acid ester)

also between individuals of the same species Vitamin K appears to interfere with this process in such a way that the dicumarol is displaced from the liver more quickly Depending upon the animal species the labeled material is excreted either in the feces or re absorbed and excreted by the kidneys The nature of the product excreted is now under investigation It seems not unreasonable to expect that it will be analogous to the metabolic product of tromexan described by Pulver and von Kaulla[1] involving opening of one lactone ring

Since this group is for the purpose of discussing possible lines of development rather than listening to reports of finished re searches I will indicate our present views as to the significance of these results There now exist several lines of investigation regarding this group of drugs the evidence of which requires reconciliation viz (a) the difference in results obtained on single dose compared to multiple doses with phenylindanedione and (b) results of

liver before extraction was equivalent to 0 86 mg The close agreement between these 2 figures is direct evidence that the radioactivity found in the liver following the injection of radioactive dicumarol is essentially all in the form of unchanged dicumarol. This means that for the liver, the radioactivity gives us a reasonably accurate measure of the dicumarol present. It may be advisable to stress the critical nature of this test The dicumarol was recrystallized 5 times with a 90% loss so as to allow optimal conditions for removal of material not dicumarol, yet the ratio of radioactivity to dicumarol was the same as in the original liver

In a further test dicumarol isolated from liver (6 1 mg of specific activity 4 1 registers per min per mg) was converted to the diacetate Specific activity of the diacetate was 3 3 (found), 3 3 (calculated) registers per min per mg and the melting point (corr) was 250 252° C (mixed M P 250 252 C)

It has been shown by a number of workers that simultaneous administration of dicumarol and vitamin K reduces the radioactivity of dicumarol A series of mice were therefore injected with 0 25 mg dicumarol together with 1 mg of vitamin K (2 methyl 1 4 naphthoquinone phosphoric acid ester synkavite) It was found that with the exception of the liver there was no significant difference in the activity found in tissues from these animals compared to mice receiving radioactive dicumarol without vitamin K at the same time The radioactivity found in the liver is shown in Figure 16 It can be seen that the radioactivity 20 minutes and 1 hour after the injection was the same for the mice which received vitamin K as for those which did not However after the first hour the animals receiving vitamin K showed much less radioactivity in the liver suggesting a more rapid displacement of dicumarol from the liver in the presence of vitamin K Some dicumarol remained in the liver after 18 hours at which time the values were about the same as for the animals without vitamin K

A number of interesting conclusions may be suggested from these results It is evident that on administration dicumarol is carried to the liver and a considerable portion remains fixed there for some time This period varies with the animal species Thus in mice the dicumarol remains in the liver for 12 hours and largely leaves by the 18th hour while in the rabbit it remains for 3 days and 7 days elapse before it has completely disappeared Presumably this may be one cause of variations in the effectiveness of prothrombopenic agents not only between different animal species but

increase in prothrombin time suggesting these were less potent than dicumarol However the rise in prothrombin time occurred much sooner for the peak was reached in 24 hours with both these substances The prothrombin time returned to normal on the second day with phenylindanedione and on the fourth day with compound 42 α hydrindone in a 50 mg dose was slightly effective on the single dose test

For the repeated dose test the drugs were given once a day for 6 days with the exception of phenylindanedione which was given in 3 doses in the 24 hours The daily intake was 5 mg per kg for dicumarol and compound 42 50 mg per kg for phenylindanedione and 12 5 mg per kg for α hydrindone The α hydrindone had no effect on the prothrombin time The other 3 drugs caused a marked increase in the prothrombin time The prothrombin time after dicumarol and phenylindanedione was more than 1 minute making it impossible to compare the activity of these drugs However it is evident that compound 42 was less effective than dicumarol in repeated doses at the same dose level Marked differences in the initial rise in prothrombin time was observed This rose rapidly with phenylindanedione less rapidly with dicumarol and relatively slowly with compound 42 Recovery was not in reverse order It was rapid with phenylindanedione (40 hours) less rapid with compound 42 (5 days) and recovery was so slow with dicumarol that death from hemorrhage occurred before recovery took place

It is evident that the dosage schedule adopted makes a great difference in the relative prothrombopenic potency assigned to these compounds It should be added that an important factor in turn modifying the multiple dose test is the animal species Thus the effectiveness on the multiple dose test of phenylindanedione is increased in dogs if the dosage interval is shortened to 8 hours but this may not be significant in man The difference in the rate of rise of the prothrombin time with these drugs will be discussed later

A relationship between the action of these drugs and their blood levels has been suggested by von Kaulla Figure 18 shows in rabbits the blood levels of various prothrombopenic agents Values for dicumarol and tromexan 50 mg per kg are from Pulver and von Kaulla The dicumarol and tromexan were determined by the colour reaction obtained for these compounds with diazotized p nitroaniline Values for phenylindanedione measuring the extracted phenylindanedione by its light absorption at 460 mμ are from my own laboratory The values for dicumarol (5 mg per kg) measured by

determination of blood levels of phenylindanedione, tromexan, and dicumarol I will review these briefly

The first point arises from our studies on phenylindanedione I have already shown that this drug is a relatively poor prothrombopenic agent when measured by the effect of a single dose similar to the assay method established by Link for dicumarol However, when the assay method is modified by giving multiple doses, this compound proves to be as effective as dicumarol In Figure 17 is shown for comparison the prothrombopenic effect in rabbits of four drugs phenylindanedione compound 42 (a sample supplied by Dr Link) α hydrindone (prepared by Chas E Frosst & Co) and dicumarol A single dose of 5 mg per kg of dicumarol had a definite effect on the prothrombin time Fifty milligrams per kilogram of phenylindanedione and of compound 42 caused the same

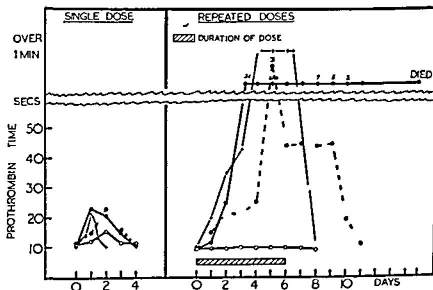


FIGURE 17

Comparison of Effect on Prothrombin Time of Single and Multiple Doses of Prothrombopenic Agents

Single dose

●———●	Dicumarol	– 6 mg
●– – – –●	Compound 42	– 50 mg
+———+	PID	– 50 mg
o———o	Hydrindone	– 50 mg

Repeated dose

●———●	Dicumarol	– 5 mg per kg per day
●– – – –●	Compound 42	– 5 mg per kg per day
+———+	PID	–167 mg per kg per day
o———o	Hydrindone	–12.5 mg per kg per day

Brodie[2] have shown that no renal excretion of unchanged dicu marol occurs as is also indicated by our results with C^{14}-dicumarol. Miss Scroggie has similarly shown this for phenylindanedione In the case of these 2 compounds the difference is related to differences in rate of metabolism alone

Therefore I believe that we must turn to the liver as the organ chiefly responsible for the difference in results obtained with dif ferent anticoagulants The experiments of Lupton[3] have shown that after dicumarol treatment of rats the liver fails to secrete prothrombin into blood perfused through it and this provides us with the first definite evidence that the action of dicumarol is exerted *through* the liver The studies with labeled dicumarol (Figures 14 15 16) show that after a single dose dicumarol remains in the liver for a considerable period of time In the mouse it remained in the liver for 16 hours and the prothrombin time re mained elevated for 3-4 days in the rabbit dicumarol remained in the liver for 3 days and the prothrombin time was elevated for 9 days vitamin K shortened the duration of dicumarol in liver and shortened the elevation of prothrombin time Hence the time that the increase in prothrombin time is maintained appears to corre spond to the time the dicumarol remains in the liver Further our results provide the first definite evidence that it is dicumarol itself which acts *in* the liver It does not seem too wild speculation to suggest from this that the factor determining prothrombopenic activity as determined by the single-dose test is the length of time the drug remains in the liver In a multiple-dose test this factor will be reduced to a minimum and we may then determine the activity of the drug in terms of ability to inhibit prothrombin formation in the liver If this be so the large series of compounds tested by Link[4] were tested for fixation in the liver not for prothrombopenic activity as related to the enzyme systems involved Link showed that substitution in almost any position in the dicumarol molecule reduces prothrombopenic activity on the single-dose test However this may be due to increased water solubility resulting in more rapid excretion by the kidney or more rapid metabolism by the liver or to complete insolubility in water so that the compound is not absorbed.

In order to limit the number of possible variables in our experi ments the radioactive dicumarol used was administered intra venously It is evident from the recent report of Weiner Axelrod Shapiro and Brodie that rate of absorption of dicumarol from the intestinal tract is another important variable They report that the

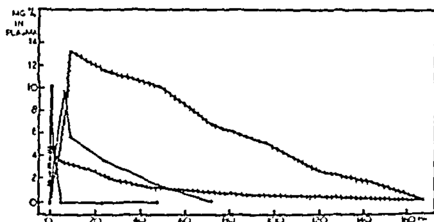


FIGURE 18

Blood Levels of Prothrombopenic Agents in Rabbits

●-/////-● Dicumarol – 50 mg per kg. per os
o-/////-o Tromexan – 50 mg per kg per os data from Pulver and von Kaulla.
o———o Dicumarol – 5 mg per kg intravenously
●———● PID – 10 mg per kg intravenously

radioactivity are shown for comparison Weiner, Axelrod Shapiro and Brodie[2] have recently described a method for determination of dicumarol based on light absorption at 315 mμ Unfortunately, data obtained with this method were not available It should be pointed out that all these methods measure the metabolic products as well as the prothrombopenic drugs given However judging from von Kaulla s data on tromexan and our data on radioactive dicumarol excretion of the metabolic products of these drugs is rapid and the amounts in the blood remain small so that we can assume that these values represent the unchanged drug However, for phenylindanedione this assumption is not justified since Miss Scroggie in my laboratory has detected significant amounts of metabolites of this compound in the blood Measurement of pheny lindanedione alone would result in a steeper curve (more rapid disappearance) than shown in Figure 18

It can be seen that, following the same single dose of the drug its rate of disappearance from the blood stream is markedly different with these different drugs In the case of tromexan Pulver and von Kaulla have shown that this is partly due to renal excretion Of 9 22 gm of tromexan administered to rabbits Pulver and von Kaulla recovered 1 37 gm unchanged in the urine 3 7 gm as metabolic products However Weiner Axelrod Shapiro and

Brodie[2] have shown that no renal excretion of unchanged dicu marol occurs as is also indicated by our results with C^{14} dicumarol Miss Scroggie has similarly shown this for phenylindanedione In the case of these 2 compounds the difference is related to dif ferences in rate of metabolism alone

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In order to limit the number of possible variables in our experi ments the radioactive dicumarol used was administered intra venously It is evident from the recent report of Weiner Axelrod Shapiro and Brodie that rate of absorption of dicumarol from the intestinal tract is another important variable They report that the

rate of absorption is slow and varies with both the individual and the dose Presumably this is also an important variable in comparing different compounds since von Kaulla reports that tromexan is absorbed more rapidly than dicumarol This factor also should be assessed in studying these compounds The differences in the initial rate of rise of the prothrombin time after dicumarol phenylindanedione and compound 42 shown in Figure 17 may be ascribed to this factor

With regard to the active groups involved in the inhibitory activity in the liver at present we know only that the following compounds are active dicumarol tromexan compound 42 and phenylindanedione (structural formula given in Figure 19) From the preceding discussion it is evident that the factors on which the effectiveness of a prothrombopenic drug depends are (a) its rate of absorption in the intestinal tract (b) its rate of metabolism by the liver, (c) its affinity for liver cells (its affinity and inhibitory power for certain enzyme systems?) All 3 factors depend on the chemical structure and properties of the drug on the one hand and

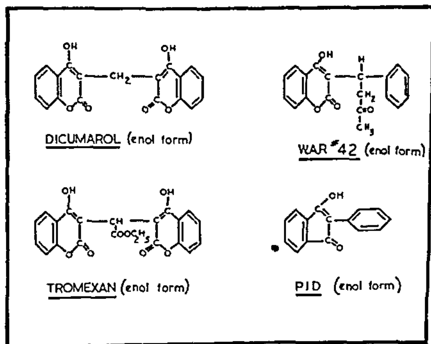


FIGURE 19
Active Prothrombopenic Agents

on biological factors which vary with the animal species on the other As a result the determination of the relative activity of prothrombopenic agents is not a simple matter and negative results without extensive testing do not provide sufficient evidence to say that a given chemical structure has no activity in the liver However by controlling the factors indicated it is hoped to determine the fundamental chemical structure necessary for dicumarol like action in the liver cell

A final point is that these investigations have demonstrated the considerable variation between different species in the biological factors which determine the action of dicumarol and similarly acting compounds On biological principles this suggests that some variation may therefore be expected in this regard within a single species such as man

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DISCUSSION

Alexander Was there any demonstrable radioctivity in the bone marrow after the administration of radioactive dicumarol?

Jaques We did a few bone marrow examinations and could not find any increase over the small residual activity found in all parts of the body presumably due to the activity in the blood

Alexander Is it correct to interpret from your data that the prolonged effect of dicumarol on the prothrombin time in contrast to the relatively rapid rate of dicumarol excretion may be related to the products of deterioration which also are prothrombopenic I am thinking particularly along the lines of the clinical experience that in liver disorders and kidney disorders the effect of dicumarol is apparently enhanced

Jaques At the moment I would not care to go that far in interpreting the data As soon as we obtain these metabolic products we plan to try them directly and see what happens That will answer

the question one way or the other I don t feel that we should at this time try to draw such conclusions from the available data

I may add that von Kaulla and co-workers have tried their tromexan, A and B and found both substances inactive I do not mean of course to say that this is necessarily true of the metabolites of dicumarol That remains to be seen

SUMMARY OF EXPERIENCES WITH THE ANTICOAGULANT, PARITOL (32 CASES)

WALLACE SORENSON and IRVING S WRIGHT

Department of Medicine Cornell University Medical College

GENERAL

Observations have been made on the administration of paritol (polysulfuric ester of polyanhydromannuroic acid) in 32 cases The ages of the cases ranged from 20 to 70 years Twenty nine of the cases were men and the other three were women Four of the cases were normal controls 28 had a variety of disease conditions including pulmonary infarction thrombophlebitis thromboangiitis obliterans axillary vein thrombosis carcinoma of the lung arteriosclerosis obliterans myocardial infarction and diabetes mellitus In 14 cases the drug was used therapeutically because of some thromboembolic disease· in the other 18 cases it was given to observe its effect.

The effect of the drug on the coagulation mechanism has been followed by a modification of the Lee-White clotting time After careful venipuncture the tourniquet is released and two or more milliliters of blood are drawn into a syringe this blood and syringe are discarded Then 5 ml of blood are drawn into a dry silicone-coated syringe and 1 ml placed in each of three 8 x 75 mm dry glass tubes Each tube is tipped gently at one-minute intervals and the end point taken as the point at which the tube can be inverted with the clot holding an unbroken surface Timing is started when the blood is drawn into the siliconed syringe The recorded clotting time is the average of three tubes By this method the average clotting time for 47 control determinations was 9 minutes Careful adherence to this technique was maintained all blood specimens were drawn by the same individuals and all the clotting times were done by one of two individuals in order to obtain the maximum accuracy possible with this method

RESULTS OBTAINED ON CLOTTING TIMES

Single doses Table IV shows the results obtained by successive determinations of the clotting time after single doses of 5 mg per kg of 10 percent paritol solutions The clotting time values are averages from 21 cases

TABLE IV

Time After Paritol	Clotting Time
Control	9 min
30 min	55 min
2 hours	38 min
4 hours	26 min
6 hours	20 min
8 hours	16 min
10 hours	11 min
12 hours	13 min

Observations obtained at the beginning of the study with 1 percent and 5 percent paritol solutions in doses up to 4 mg per kg showed curves with clotting times only up to 20 minutes Two later curves obtained with 2 mg per kg doses of 10 percent paritol solution showed the average maximum clotting times of 39 minutes

Repeated doses Amounts of 2 mg per kg of 10 percent solution were given to 6 patients during maintenance therapy The average values of 22 curves are shown in Table V

TABLE V

Time	Clotting Time
Before any paritol given	9 min
Before next dose of paritol	14 min
30 min after paritol	50 min
8 hours after paritol	15 min
12 hours after paritol	15 min

TABLE VI

Time	Clotting Time
Before any paritol given	9 min
Before next dose of paritol	13 min
30 min after paritol	55 min
10 hours after paritol	18 min
12 hours after paritol	16 min

Amounts of 3 mg per kg of 10 percent solution were given to 3 patients during maintenance therapy The average values of 7 curves are shown in Table VI

Amounts of 5 mg per kg of 10 percent solution were given to 5 patients during maintenance therapy The average values of 19 curves are shown in Table VII

TABLE VII

Time	Clotting Time
Before any paritol given	9 min
Before next dose of paritol	12 min
30 min after paritol	69 min
8 hours after paritol	21 min
12 hours after paritol	15 min

TOTAL AMOUNTS OF PARITOL ADMINISTERED

Eleven patients have been maintained on continuous paritol therapy for periods over 24 hours The longest period of continuous administration has been 8 days in 2 cases The largest amount of the drug given to any one patient was 3 440 mg in a 5 day period

REACTIONS TO PARITOL

One immediate severe reaction was observed in one patient after a dose of 5 mg per kg (315 mg) of 1 percent solution The reaction lasted about 30 minutes and was characterized by signs of vascular collapse vomiting and defecation Treatment given was oxygen by mask and epinephrine subcutaneously This patient was found to have had similar reactions to other medications given intravenously including papaverine a digitalis preparation and magnesium sulfate

Three patients 30 minutes following paritol showed a peculiar reaction consisting of the rapid development of edema of the hands and feet associated with pricking sensations in the edematous parts This edema lasted 6 to 10 hours There was no associated generalized urticaria nor was there respiratory distress

One patient with an elevated blood urea nitrogen secondary to probable chronic glomerulonephritis showed a further rise of the blood urea nitrogen after repeated doses of paritol One other patient who had a normal blood urea nitrogen on admission had a rise in blood urea nitrogen during paritol therapy However this

patient was also getting daily thiomerin injections which could have played a role in this rise

One patient following paritol administration had two episodes characterized by increased respiratory distress However, it was felt that at least one of these and maybe both, were due to repeated pulmonary emboli

One patient who was nauseated on admission seemed to have increased nausea and vomiting after paritol was given. However after his initial nausea had subsided repeated doses of paritol were given without causing any distress

In one patient a small extravasation of paritol outside the vein produced a rather marked reaction of pain, swelling increased heat, and redness which lasted about 24 hours No slough occurred

EFFECT OF PARITOL ON OTHER LABORATORY DETERMINATIONS

Prothrombin Times Following paritol the prothrombin time was prolonged slightly and returned to normal in approximately the same time as the clotting time

RBC and WBC No change has been observed in these values that could be attributed to paritol

Platelets No gross changes in the number of platelets have been observed

Kidney Function The rise in blood urea nitrogen has been noted above No other abnormalities of the urine have been observed

Liver Function One patient had a further slight rise in elevated thymol turbidity and cephalin flocculation values during the period he was receiving paritol Whether these changes were due to this drug appears questionable No other definite evidence of liver damage was observed

CONCLUSIONS FROM OBSERVATIONS TO DATE

It appears that paritol is effective enough in prolonging the clotting time of the blood to be useful in the treatment of thromboembolic diseases Its usefulness will probably be found in short term treatment where immediate action is desired and in the period before dicumarol begins to be effective in prolonging the prothrombin time

The observations indicate that dosage schedules of 2 mg per kg to 5 mg per kg every 12 hours give clotting time curves within the range thought to be effective in the treatment of thromboembolic conditions

The action of pantol over an 8- to 12 hour period is a definite advantage over heparin and significantly decreases the inconvenience of intravenous anticoagulant therapy

Some reactions to pantol have been observed The slow administration of the drug is worth emphasizing so that some of the first symptoms may be observed and the appropriate measures instituted Extravasation of the drug outside the vein should be avoided

Contraindications to the use of pantol now appear to be impaired renal function particularly where there is any urea retention as well as the usual contraindications of any type of anticoagulant therapy

DISCUSSION

Barker Do you recommend the slow injection of a concentrated or a dilute solution?

Wright I would favor giving it in dilute form although we do not as yet know the most satisfactory concentration to use

Barker Did any of the patients bleed after they received pantol and is protamine an antagonist to pantol?

Wright The answer to the first question is that we have had no bleeding In answer to the second we do not know if protamine is a satisfactory antagonist

There is one other advantage the cost of pantol will be approximately one fifth that of heparin. When heparin costs $15-$26 a day and you can reduce the cost one fifth it is something worthy of consideration in the economics of medicine Pantol is a derivative of alginic acid and easy to produce

Barker What would you do if a patient developed severe bleeding one hour after you had given an injection of pantol?

Wright So far that has not happened but we are prepared to give transfusions

Allen Will you elaborate more fully on how the clotting time varies between the first and eighth hour after the administration of pantol?

Wright The return of the clotting time to normal is two or three times slower with pantol than with intravenously administered heparin It resembles more the change observed with the so-called depot heparin

Seegers Has the preparation of this compound been described in the literature?

Wright Drs J Seifter and A J Begany (Studies of a Synthetic Heparinoid *Am J Med Sc* 216, 234 (1948)) have published some details

Tocantins When Dr Seifter presented work on this anticoagulant about a year or so ago at the Physiological Society in Philadelphia he followed the blood of some rabbits every hour or half hour after administration of the drug and showed that after the coagulation time came back to normal there was a period of hypercoagulability I wonder whether that also occurs in the blood of these patients or controls

Wright There is no evidence of it in our experience even in patients followed for periods of as long as 8 days

Jaques Regarding the price problem with heparin my understanding is that the difficulty is not in preparing the heparin *per se* but in preparing a product for parenteral use It is the cost of the tests used in standardizing the product and the tests for toxicity pyrogens etc which keeps the price of heparin high The actual cost of the isolation of heparin from lung or other tissue is a small portion of the total cost It seems to me the same problem will exist with this new compound Any product that is going to use a biological source to start with in contrast to the material prepared synthetically by the chemist offers much more in the way of problems to the industrial manufacturers in terms of insuring absence of pyrogens, etc

ZETA POTENTIAL MEASUREMENTS AS A TOOL FOR STUDYING CERTAIN ASPECTS OF BLOOD COAGULATION

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It is well known that solid surfaces exert a marked effect on the coagulation of blood although the mechanism by which they do so is not at all understood The existing information was I think well summarized and explored at the second Macy Foundation Conference last year particularly by Dr Barker[1] It is scarcely necessary at this time to review the considerable volume of reported experiments on rates of clotting as influenced by the wall of the containing vessel other than to call attention to the fact that those surfaces which appear to preserve blood from coagulation are in general substances which are nonpolar in character and not wettable by water The works of Jaques[2] on silicone coated vessels of Hirschboeck[3] on collodion lined vessels and of Freund[4] as early as 1886 on vessels coated with paraffin or vaseline may be cited as examples of the marked delay brought about in the clotting time as compared with that in glass containers In contrast to these and other organic materials glass is a substance which has strongly polar or electrovalent bonds between its constituent atoms even the least polar being about 50 percent electrovalent (cf Pauling[5]) This polarity gives rise to strong electrical forces in the neighborhood of the surface of the solid surface in contact with solutions and to this polarity may be ascribed a variety of surface phenomena such as preferential adsorption of polar substances or the occurrence of electrical charges at interfaces between solids and liquids The latter effect is undoubtedly an outgrowth of the former These charges give rise to a potential difference between the interface of the glass and the liquid and the interior of the liquid in the solid liquid systems known as the zeta potential which is precisely measurable in a large variety of systems The zeta potential is the

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only known property of solid surfaces which can produce long range repulsive forces in water solution By that I mean forces effective at a distance of more than a few Angstrom units Rather extensive mathematical theories of these forces have been developed by Langmuir[6], Verwey[7], and others, and there is much experimental evidence, although largely qualitative, to confirm these theories It has generally been believed that the zeta potentials of paraffin or other organic surfaces are very much lower than those of polar surfaces and consequently electrical effects in the neighborhood of these organic surfaces should be much less pronounced It seems reasonable to suppose that the different speeds of clotting are ascribable to differences in the solid surface, and quite possibly even to differences in the zeta potential Indeed, Gortner and Briggs[8] have postulated that the underlying factor is very likely connected with a difference in the zeta potentials of the different materials and as evidence they cited that the zeta potential of glass is about −30 millivolts, while that for paraffin coated glass was zero according to their measurements They believe that the high zeta potential of glass would favor adsorption of positively charged colloids at the glass blood interface thereby concentrating an essential material which initiates the clotting process Since they found the zeta potential of paraffin in contact with water to be zero they reasoned that blood should not clot in paraffin lined vessels

Other evidence indicating that attention might well be directed towards the solid surface may be mentioned It is well known that blood platelets appear to play a prominent part in coagulation although their role is probably not established It has been proposed that thromboplastin may be liberated from the platelets although platelet free blood has been observed to clot A high concentration of platelets has also been observed in the initial region of the clot *in vivo*, so we may say it is perhaps significant that platelets represent solid surfaces and the possibility of surface effects around the solid therefore enters the picture

It is also found that clot formation *in vivo* is frequently and perhaps always associated with injury such as accidental trauma surgery, infection or new growths to the blood vessel walls However blood in a tied off but uninjured vessel will remain for a long time without clotting Here again the nature of the solid liquid interface apparently plays a part

In addition to the surface electrical effects which may play a prominent role in blood coagulation it is conceivable that the

function of anticoagulants is partly of an electrical nature since Chargoff and co workers[9] and Wolfram[10] have reported that heparin for example behaves in solutions as a highly charged anion and migrates in electrical fields towards the anode These considera tions which I have mentioned briefly led Dr Wright to set in motion this investigation with the expectation that a study of the zeta potentials of glass and silicone coated surfaces in contact with blood or fractions of blood would reveal much interesting informa tion

It is planned eventually to extend the investigation of the measure ments of zeta potentials to the solid particles such as blood platelets present in blood

MEASUREMENT OF THE ZETA POTENTIAL

In these experiments the surfaces which were studied were capillary walls which although cylindrical in shape may actually be considered plane surfaces from a molecular point of view be cause the curvature of course is very large compared with mole cular sizes To make clear what is being measured and what is meant by the zeta potential the variation of the electrical potential with distance from a solid surface is shown in Figure 20 A solid surface generally is electrically charged and the mechanism by which the charges arise on the surface is not perfectly understood It is pre sumably a result of selective adsorption of certain ions from solution and possibly also hydration of the surface and the resulting ioniza tion of water molecules by hydration In special instances polar groups attached to the surface such as carboxyl groups may ionize and so create a charged surface The charges on the solid in contact with the electrolyte solution produce the potential given by the solid curve which gradually diminishes in magnitude with increas ing distances depending on the concentration and type of ions in the solution As a result of the zeta potential usually negative the ions in the solutions will not have the same concentration in the neighborhood of the wall as they do in the solution The positive ions will be attracted toward the wall and the negative will be re pelled in the situation shown and their concentrations ($n+$ and $n-$) will vary in the general way shown by the dotted lines Far from the wall the potential will approach zero and the ion concentra tions will be that of the bulk solution The zeta potential is simply the difference between the wall potential and that of the interior of the solution arbitrarily taken as zero Knowing the zeta potential and the ion concentration it is possible to solve completely all the

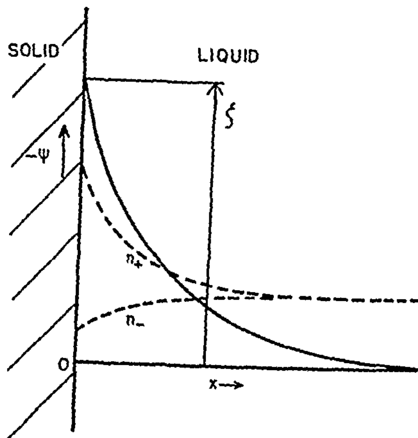


FIGURE 20
Relative variation with distance from surface of the electric potential (solid curve) and the concentrations of positive and negative ions (dashed curves)

variables of the system through the use of the Poisson Boltzmann equation, for this particular instance

The zeta potential is most accurately measured by producing streaming potentials which result when a liquid is forced through a capillary Figure 21 shows schematically why the streaming potential arises in such an experiment When the liquid flows through the tube indicated in lengthwise section the positive ions present in excess in the solution are carried along in the direction of flow and give the efflux end of the tube a positive charge As this charge builds up a flow of electric current back through the tube begins since the solution is a conductor (only the excess positive ions in the solution are shown in Figure 21) When the tendency of the liquid flow to sweep the positive ions of the electric double layer along the tube is exactly counterbalanced by the counter flow of

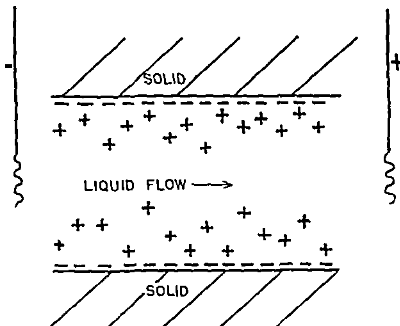


FIGURE 21

Schematic diagram of capillary in lengthwise section showing negative charges on wall and positive charges in solution

the ions under the influence of the electric potential difference a steady potential difference between the ends of the tube results known as the streaming potential. Under proper experimental conditions the streaming potential is related to the zeta potential by the equation of Helmholtz

$$\zeta = \frac{4\pi\eta E\kappa}{DP}$$

where η is the viscosity coefficient, κ is the specific conductance of the liquid in the capillary, E is the streaming potential when the liquid is forced through under pressure P (nitrogen was used here to apply the pressure) and D is the average dielectric constant of the liquid in the ionic double layer. From this equation it is evident that the apparatus required must include devices for measuring the streaming potential, the pressure and the electrical conductivity of the liquid in the capillary. In this work the viscosity and dielectric constant were assumed to be the same as that of water so that $\zeta = 9.662 \times 10\ \kappa E/P$ mv at 25.0° C. No attempt was

made to control the temperature rigidly since it has been shown that the temperature coefficient of the zeta potential of aqueous systems can be ignored in streaming potential measurements made under ordinary laboratory conditions (approximately 25° C.).

The apparatus* used was essentially that described by Jones and Wood[11] and consisted of two principal units, namely one for the measurement of the streaming potential at a known pressure and the other for the determination of the electrical conductivity of the solution in the capillary. A general view of the complete streaming potential apparatus is shown in Figure 22 (the more sensitive

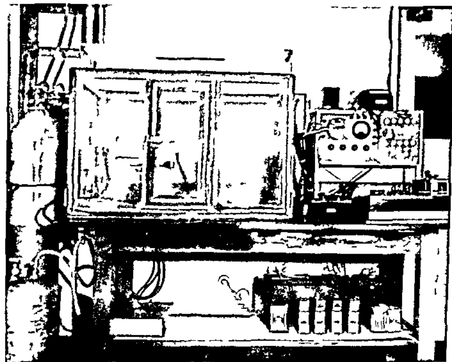


FIGURE 22
Apparatus for production and measurement of streaming potential

L and N Type E galvanometer, however, was substituted for the one shown in the photograph).

The potential difference between the electrodes was measured

* Considerable preliminary work on the development of the apparatus was carried out by Commander F. G. Hirsch, USN (MC), who was formerly a member of the Vascular Laboratory of Cornell University Medical College. We wish to express our appreciation for his contributions to the work described here.

with an L and N Type K potentiometer The proper choice of the null instrument is critical here because the electrical resistance of the instrument must be large compared with the resistance of the cell being measured For ordinary potential measurements a galvanometer is satisfactory but for streaming potential measurements an instrument with a higher internal resistance is necessary This requirement is satisfied by the L and N thermionic amplifier (Cat No 7673) which has a resistance of at least 10 ' ohms and is sensitive to a potential difference of about 0 2 mv The entire apparatus was shielded with copper screen and the connecting wires were shielded to prevent induced charges and electrical leakage For the measurement of the pressure of the nitrogen a mercury manometer was used A 15 gallon tank was employed as a ballast reservoir for the high pressure while the other side was open to the air when flow was taking place A pressure of about 40 cm of mercury was used for the measurements reported in this work Stopcock and electrical switch arrangements permitted measurements to be made when the liquid flowed in either direction

For the determination of the specific conductance it was necessary to measure the resistance of the solution in the capillary by means of a high resistance direct current bridge built into the streaming potential apparatus This bridge was capable of measuring resistance in the range of 10 to 10 ohms with a precision of 0 5% or better A decade resistance box adjustable in steps of 1 ohm from 1 to 10 000 ohms was used as the variable resistor The capillary a precision resistor of 1 megohm and a precision resistor of 0 1 megohm constituted the other three resistances of the bridge network Thus the specific conductance of the capillary solution could be determined after having determined the cell constant of the capillary (using a standard solution such as 0 01 N KCl) according to the usual equation $\eta = (L/\pi r\ )/R$ where η is the specific conductance $(L/\pi r\ )$ is the cell constant and R is the measured capillary resistance

The cell consisted of two glass reservoirs each of about 20 ml capacity connected by the capillary tube as illustrated in Figure 23 Pressure applied by nitrogen through either side arm of the reservoir forced the solution from one side to the other On the top of each reservoir is a glass cap through which is sealed a platinum wire which extends to the bottom of the reservoir On the lower end of the wire is deposited a porous mixture of silver and silver chloride which is a nearly insoluble reversible electrode The capillary shown was 6 9 cm in length 0 0204 cm in bore and its cell

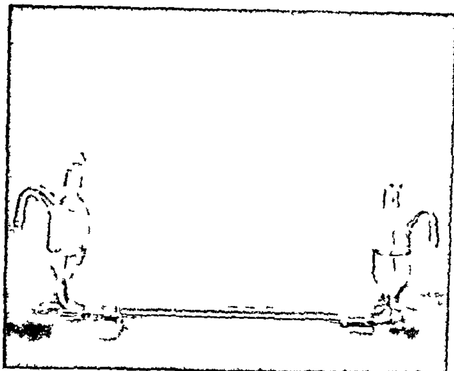


FIGURE 23
Capillary reservoirs and electrodes assembled

constant was 21 000 cm^{-1} The silicone coated capillary was prepared by treating with G E Dri film (9987) several times before using it was 14 6 cm in length 0 0372 cm in bore and its cell constant was 13 400 cm^{-1}

Alexander What kind of glass?

Wood This capillary in Figure 23 is vitreous silica not ordinary glass We think silica was a better material to use since it was a pure substance and presumably would be more reproducible In the capillary which was coated with silicone pyrex glass was used since it was felt that there the type of glass would make no difference the surface presumably being a silicone surface One of the interesting things that was observed first of all when streaming solutions of potassium chloride or a phosphate buffer through the capillary was that the silicone coated capillary did not give a zero zeta potential as shown in Table VIII

With 0 001 molar potassium chloride one zeta potential measurement gave −68 mv for the silica and −44 mv for the silicone

TABLE VIII

ZETA POTENTIALS OF SOLUTIONS IN VITREOUS SILICA AND SILICONE COATED CAPILLARIES

	Zeta Potential (Mv)	
Solution*	Silica Capillary	Silicone-Coated Capillary
0 001 M KCl	−68	−44
0 01 M KCl	−52	−25
KH PO$_4$ KOH (approx 0 001 M)	−80	−61

* The specific conductances at 25 0 C of the three solutions were 0 001 M KCl, 149 x 10^{-6} mhos cm 0 01 M KCl, 1430 x 10^{-6} mhos cm^{-1} and KH_2PO_4-KOH 118 x 10^{-6} mhos cm The pH of the KCl solutions was 5.5 KH_2PO KOH 6 9

coated capillary In the phosphate buffer which was usually used as the standard solution the silica capillary gave a zeta potential of −80 mv and the silicone-coated about −61 So the zeta potential of the silicone coated capillary was around two thirds to three quarters the magnitude of the zeta potential of the glass capillary I have not been able to get agreement with Briggs and Gortner that the zeta potential of organic surfaces is zero I have also found that paraffin polystyrene and paraffin coated polystyrene are actually about the magnitude of glass which is rather surprising

Edsall You got about −80 with glass?

Wood Yes with 0 001 molar phosphate buffer pH 7 and −60 for silicone with the same solution

EFFECT OF pH ON THE ZETA POTENTIALS

One of the things which was discovered rather early in the work was that the pH had a considerable effect on the zeta potential which of course comes as no surprise to anyone who has dealt with protein solutions Surfaces in contact with a protein solution generally become coated with the protein and thereby exhibit properties characteristic of the protein Since the present work is primarily concerned with the zeta potential of plasma proteins and anti coagulants such as heparin it is necessary to have some knowledge about the effect of pH on the potentials A description of the influence of pH on very dilute solutions of blood is given in Table IX and Figure 24 It is recognized that the measurements made at the different pH values were not carried out at constant ionic strength but it was found that the changes in zeta potential due to a pH

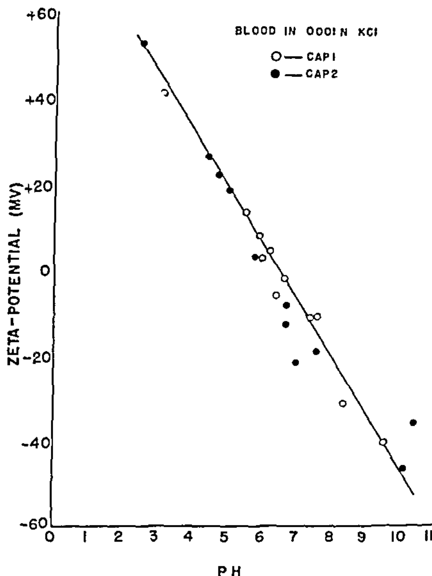


FIGURE 24

change outweighs that due to a small variation in ionic strength But the pH must be fixed in order to be able to investigate the effect of heparin on the zeta potentials of the plasma proteins

Table X and Figure 25 show the change with pH of dilute solutions of heparin in 0 001 M KCl

One of our particular interests in this work was to see whether effectiveness *in vitro* of anticoagulants has any relationship to the

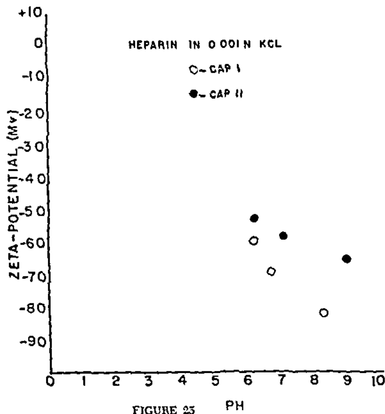


FIGURE 25

TABLE IX

THE EFFECT OF pH ON DILUTE SOLUTIONS OF BLOOD IN 0 01 N KCl

Silicone Coated Capillary (II)		Vitreous Silica Capillary (I)	
pH	Zeta Potential (Mv)	pH	Zeta Potential (Mv)
3 0	+42	2 9	+54
5 4	+14	4 3	+27
5 8	+ 9	4 6	+23
5 9	+ 2	4 9	+20
6 1	+ 6	5 7	+ 4
6 3	— 5	6 6	— 7
6 5	— 1	6 6	—12
7 3	—10	6 9	—21
7.5	—10	7.5	—18
8 3	—36	10 1	—46
9 5	—41	10 4	—36

* 0 03 ml. of blood per 100 ml. of 0 001 M KCl small amounts of dilute KOH and HCl solutions were added to change the pH of the standard KCl solution (pH 5 5)

TABLE X

THE EFFECT OF pH ON FIBRINOGEN AND HEPARIN IN KCl SOLUTIONS

Additive Amt /100 ml 0 001 N KCl	Vitreous Silica Capillary		Silicone Coated Capillary	
	pH*	Zeta Potential (mv)	pH*	Zeta Potential (mv)
None	5 5	—68	5 5	—44
None	6 2	—69	6 5	—62
5 mg fibrinogen	6 0	— 1	6 0	— 9
5 mg fibrinogen	7 0	—13	7 3	—26
5 mg heparin	6 2	—60	6 2	—52
5 mg heparin	6 7	—69	7 1	—58
5 mg heparin	8 3	—82	9 0	—65

* *To increase the pH above 5.5 (standard solution) small amounts of dilute KOH were added to the 0 001 N KCl solution*

zeta potential of the vessel walls We were also anxious to see whether we could relate to the zeta potential, the question of why blood clotted rapidly in a silica or glass tube and much more slowly in a silicone tube

Ferry How do the two capillaries differ?

Wood The black dots in these Figures 24 and 25 were data from the silicone coated and the open circles represent silica capillary using both capillaries I might say in advance that no marked difference was ever observed between the two with respect to zeta potential

THE EFFECT OF HEPARIN ON THE ZETA-POTENTIAL OF BLOOD

Several experiments were performed using a small amount of whole blood dissolved in the 0 001 N phosphate buffer solution and the zeta potentials were determined before and after the addition of heparin The data in Table XI show that a small negative potential results in both capillaries when the dilute blood solution is measured at a pH of 6 9 and that the potential is made decidedly more negative by the addition of heparin Moreover it is seen that the heparin blood potential which is intermediate between that of the blood and heparin alone is approximately the same irrespective of whether the blood is first added to the capillary followed with heparin or the heparin is used first followed with blood

The potential determining material coating the surface of the capillaries may be due to one or more of the plasma proteins and also the red cells themselves

TABLE XI

EFFECT OF HEPARIN ON THE ZETA POTENTIAL OF BLOOD

Additive Amt/100 ml. Phosphate	Vitreous Silica Capillary		Silicone-Coated Capillary	
	pH	Zeta Potential (Mv)	pH	Zeta Potential (Mv)
0 05 ml blood	6 9	— 6	6 9	— 8
0 05 ml blood +5 mg heparin	6 6	—45	6 6	—41
5 mg heparin	6 9	—89	6 9	—84
5 mg heparin +0 05 ml blood	6 7	—43	6 7	—51
0 05 ml washed red cells	7 1	—11	—	—

The possibility of red cells undergoing lysis was taken into account On adding washed red cells to the standard phosphate solution it was found that the zeta potential was —11 mv It is possible that the hemoglobin may have contributed something to the layer of protein presumably adsorbed on the capillary wall

Alexander You added heparin and red cells?

Wood I dont believe that we have any experiments involving heparin and red cells

Tocantins You add 0 05 ml of blood to 100 ml ?

Wood That is correct

Tocantins The blood is diluted considerably

Wood It is very substantially diluted We cannot work with any very high concentration of blood because of the fact that blood is such a highly conducting liquid that it in effect short circuits the streaming potential

Tocantins That may be one reason why not much difference is found between the values in vitreous silica and silicone because blood that is diluted to that extent would not clot at a significantly different rate in glass or silicone

Wood In fact it would never clot at all

Tocantins Relatively little dilution is needed to eliminate the different rates of clotting

Wood I would like to point out that there does not appear to be any significant difference in the behavior of the blood in the vitreous silica and silicone coated capillaries but in both cases the addition of heparin increases the charge very substantially I will have some information to present later regarding the effect of

concentration As the concentration of fibrinogen is increased there is very little effect on the zeta potential in the silica capillary

Allen The amount of heparin you have used is quite large considering the size of the blood sample Do you have any information on the effect produced by smaller quantities of heparin?

Wood There are some data later on different concentrations of heparin but in general we did use what is admittedly a rather large concentration of heparin as compared with the concentration of blood

THE EFFECT OF HEPARIN ON THE ZETA-POTENTIALS OF PLASMA AND SERUM

In addition to a study of whole blood and red cells plasma and serum were also investigated Nonclotting plasma (oxalated) and clotting plasma (prepared by centrifuging the venous blood in a *silicone coated tube) were used A portion of the clotting plasma* was allowed to clot by transferring it from a silicone coated container to glass the fibrin was removed and the resulting serum was also investigated The zeta potentials of these different systems are recorded in Table XII The nonclotting plasma gave a zeta potential of about −10 mv in both capillaries using two different concentrations of plasma Heparin increased the negative potential of the nonclotting plasma to approximately the same degree in both capillaries and at two different concentrations of heparin

TABLE XII

EFFECT OF HEPARIN ON THE ZETA POTENTIALS OF PLASMA A

Additive Amt/100 ml Phosphate	Vitreous Silica Capillary		Silicone-Coated Capillary	
	pH	Zeta Potential Mv	pH	Zeta Potential Mv
0 05 ml plasma (oxalated)	6 9	−10	6 8	−10
0 05 ml plasma (oxalated) + 5 mg heparin	6 8	−23	6 8	−23
1 0 ml plasma (oxalated)	6 8	−12	6 8	− 9
1 0 ml plasma (oxalated) + 5 mg heparin	6 8	−22	6 8	−15
0 05 ml plasma (clotting)	6 8	−14	6 7	−13
0 05 ml plasma (clotting) + 5 mg heparin	6 8	−35	6 7	−33
0 05 ml serum (no oxalate)	6 6	−17	6 6	−17
0 05 ml serum (no oxalate) + 5 mg heparin	6 4	−29	6 3	−24

The zeta potentials of the clotting plasma in the two capillaries were slightly more negative than in the case of the nonclotting plasma Likewise heparin gave a more negative effect in the case of the clotting plasma The zeta potentials of the serum were some what more negative than those of the plasma in both capillaries and heparin similarly made these potentials more negative

THE EFFECT OF HEPARIN ON THE ZETA POTENTIALS OF PLASMA FRACTIONS

The zeta potentials of several bovine plasma fractions and the effect of heparin on these potentials were determined The results given in Table XIII agree qualitatively with those obtained by electrophoresis of plasma, namely the albumin boundary moves the

TABLE XIII
EFFECT OF HEPARIN ON THE ZETA POTENTIAL OF PLASMA FRACTIONS

Additive Amt/100 ml. Phosphate	Vitreous Silica Capillary		Silicone-Coated Capillary	
	pH	Zeta Potential Mv	pH	Zeta Potential Mv
5 mg Fraction II (c188A)	6 9	+10	7 1	+ 2
5 mg Fraction II (c188A) + 5 mg heparin	6 9	–24	7 0	–24
5 mg Fraction III 1 (c155)	7 0	–14	7 0	–12
5 mg Fraction III 1 (c155) + 5 mg heparin	6 9	–29	6 9	–25
5 mg fibrinogen	6 7	–20	7 1	–24
5 mg fibrinogen + 5 mg heparin	6 6	–37	6 8	–40
5 mg fibrinogen	7 0	–16	7 1	–16
5 mg fibrinogen + 8 mg heparin	6 9	–41	7 0	–25
5 mg cryst albumin	6 8	–53	6 8	–45
5 mg cryst albumin + 5 mg heparin	6 9	–52	7 0	–49
5 mg fibrinogen + 5 mg cryst. albumin	6 8	–15	6 8	–12
5 mg fibrinogen + 5 mg cryst. albumin + 5 mg heparin	6 7	–36	6 6	–28
5 mg Fraction IV (c143)	7 1	–58	6 8	–43
5 mg Fraction IV (c143) + 5 mg heparin	7 0	–51	6 9	–49

fastest in an electric field and is therefore charged the most highly negative whereas the mobility of Fraction II (c 188 A) (gamma globulins) is the lowest and consequently is the least negatively charged

It is of interest to note that heparin makes the zeta potentials of Fraction II (c 188 A), Fraction III 1 (c 155) and fibrinogen more negative, whereas it has little or no effect on the crystalline albumin and Fraction IV (c 143) The synthetic mixture of fibrinogen and albumin shows that fibrinogen probably has a greater influence on the surface than does albumin Fraction IV (c 143), which contains 55% albumin gives zeta potentials which are practically identical with those developed by the crystalline albumin

EFFECT OF OTHER ANTICOAGULANTS ON THE ZETA-POTENTIALS

After observing that heparin charges up the surfaces in contact with fibrinogen and with blood it naturally was of interest to see if other anticoagulants did the same thing Heparin is the most potent anticoagulant known at the present time Recently however a synthetic material called paritol has been investigated

TABLE XIV
EFFECT OF OTHER ANTICOAGUANTS ON THE ZETA POTENTIALS

Additive Amt /100 ml. Phosphate	Vitreous Silica Capillary		Silicone-Coated Capillary	
	pH	Zeta Potential (Mv)	pH	Zeta Potential (Mv)
5 mg fibrinogen	6 7	–20	7 1	–24
5 mg heparin	6 7	–64	6 6	–44
5 mg heparin + 5 mg fibrinogen	6 6	–37	6 8	–40
5 mg paritol	6 7	–63	6 7	–54
5 mg paritol + 5 mg fibrinogen	6 7	–32	6 7	–29
5 mg "phosphatide inhibitor"	6 7	–69	6 8	–85
5 mg "phosphatide inhibitor" + 5 mg fibrinogen	6 7	–39	6 7	–53
5 ml 1 5 pentanodiol*	4 2	–11	4 2	–14
5 ml 1 5 pentanodiol* —0 03 ml blood	4 4	+18	4 4	+19

* 100 ml of 0 001 M KCl solution was used in place of the 100 ml of phosphate solution

clinically by Sorenson and Wright and has been found to have anticoagulant properties Overman of our laboratories has likewise isolated a phosphatide material from soybeans which inhibits the blood clotting process A comparison of paritol and the "phosphatide inhibitor" with heparin is made in Table XIV showing that the three anticoagulants behave similarly with fibrinogen in that they cause the zeta potential to become more negative You will recall that albumin which had a high negative charge as well as a high negative zeta potential −55 or thereabouts but no anticoagulant activity did not bring about this effect

EFFECT OF HEPARIN ON THE ZETA POTENTIALS OF OTHER BLOOD SUBSTANCES

The production of thrombin by the action of thromboplastin on prothrombin is considered to be one of the first steps in the blood clotting mechanism From the data in Table XV it is seen that the zeta potentials of thrombin and thromboplastin are made more negative by the addition of heparin the latter being affected more markedly than the former The data also show that protamine which counteracts the effect of heparin physiologically gives a highly positive zeta potential which becomes negative on the addition of heparin

TABLE XV
EFFECT OF HEPARIN ON THE ZETA POTENTIALS OF OTHER BLOOD CLOTTING

Additive Amt./100 ml. Phosphate	Zeta Potential (Mv)			
	pH	Vitreous Silica Capillary	pH	Silicone-Coated Capillary
5 mg thrombin	6 7	−39	5 8	−20
5 mg thrombin + 5 mg heparin	6 7	−44	6 4	−45
5 mg thromboplastin	6 7	−29	6 7	−28
5 mg thromboplastin + 5 mg heparin	6 6	−56	6 6	−35
5 mg protamine	7 0	+30	6 6	+32
5 mg protamine + 5 mg heparin	7 0	−46	7 0	−44
5 mg heparin	6 7	−64	6 6	−44

Table XVI gives further mixtures of heparin and protamine showing that sufficient protamine overcomes the negative potential of the heparin

TABLE XVI

Additive Amt./100 ml 0 001 N KCl	Zeta Potential (Mv)	
	Vitreous Silica Capillary	Silicone-Coated Capillary
5 mg heparin	—96	—66
5 mg heparin + 5 mg protamine	—64	—45
5 mg heparin + 10 mg protamine	— 1	— 1
5 mg heparin + 15 mg protamine	— 2	— 0 5
5 mg protamine	+37	+32
15 mg protamine	+29	+21
15 mg protamine + 5 mg heparin	+ 5	+ 2

Alexander May I ask at what pH s these were and whether the protamine affected this?

Wood The pH of these experiments was not measured at the time but should have been they probably were around 5 5 to 6 which would be given by the carbon dioxide of the air in the potassium chloride solution

Alexander I was just wondering whether protamine affected the pH because of its basic quality

Wood It might very well have but I suspect in these small quantities that the carbon dioxide present in the solution would be sufficient to bring it up to around 6 I really don t know the answer to that

Jaques Was that protamine sulfate which was used? In what form was the protamine?

Wood That I do not know

Jaques Solutions of protamine are sometimes quite acid since protamine is usually in the form of the sulphate The mixture might be anywhere from pH 2 5 up to pH 10 depending upon the pH of the reagent plus the effect of the dilution

Wood *Of course this is an extremely dilute solution and I doubt* very much whether the protamine had a pronounced effect on the pH of the solution

Jaques Just to correct Dr Alexanders notion it might be basic and it just as well might be acid

Wood That is quite possible These slides merely indicate that when the protamine and heparin were mixed together they ap peared to counteract their opposite effects on the charge on the wall

These experiments which I have described I should have pointed out at the beginning were all carried out by Dr Horan and not

done by me That is one of the reasons why I am not as familiar as I should be with some of the details which I have been asked about. But Dr Horan unfortunately could not come here to give the talk and I really am acting as a substitute for him and apologize for my shortcomings in that respect. He has left this laboratory to everyone s regret and has been succeeded by Dr Sheppard who is continuing the survey A few of Dr Sheppard s experiments might very well be mentioned at this point

THE EFFECT OF FIBRINOGEN CONCENTRATION ON THE ZETA POTENTIAL OF VITREOUS SILICA

To learn whether a radical change in behavior of the system might be expected with more concentrated solutions the zeta potential of fibrinogen solutions in higher concentrations up to the limit possible with the present apparatus has been measured and the results are shown in Table XVII and Figure 26 It is clear that

TABLE XVII

EFFECT OF CONCENTRATION ON THE ZETA POTENTIAL BOVINE FIBRINOGEN

Fibrinogen mg./100ml. 0 001 M KH_2PO_4–KOH buffer	pH	Streaming potential per cm Hg pressure	Specific Conductance (mhos /cm. x 10)	Zeta potential (mv)
0	7 0	6 84 mv	1.33	—89
5	6 8	1 83	1 36	—24
10	6 9	1 35	1 69	—22
25	6 8	0 85	2.12	—17
50	6 8	0 48	2.94	—14
100		0 25	4 37	—11
		HUMAN FIBRINOGEN		
0	7.5	4 07	2 03	—81
1	7 0	1.32	2.21	—28
2.5	7 0	1 09	2.16	—23
5 0	7 0	0 83	2 32	—19

some change is to be expected with the zeta potential expected to be lower probably mainly the result of the higher ionic strength which results in a decrease of the double layer thickness However it appears that the fibrinogen is so surface active that it rather completely covers the surface even at a concentration of only 1 mg

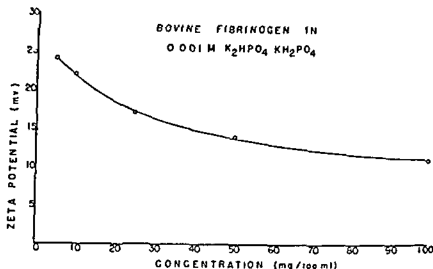


FIGURE 26

per 100 cc The magnitude of the streaming potential in the most concentrated solution shows why measurements with whole blood cannot be carried out with the present apparatus since at 30 cm pressure the streaming potential is only 7 5 mv Since it is inversely proportional to the specific conductance assuming a fixed zeta potential it would be of the order of magnitude of 0 3 mv with blood for which the specific conductance is about 0 01 mhos per cm We expect that in the near future apparatus will be available which will permit use of whole blood or of fibrinogen solutions of comparable concentration to that in whole blood

THE EFFECT OF CERTAIN PURE PROTEINS ON THE ZETA POTENTIAL OF FIBRINOGEN SOLUTIONS

Dr Sheppard had available some pure trypsin and soybean trypsin inhibitor (STI) and determined to make a test of the effect of these substances on the fibrinogen zeta potential and to attempt correlation with the clotting time He found as shown in Tables XVIII and XIX that the STI had a very high negative potential alone whereas the trypsin had a very low potential Adding the STI to the fibrinogen elevated the potential from 19 mv to 32 mv and the trypsin also elevated the potential to a value higher than either that of the trypsin or the fibrinogen In the latter experiment the possibility of proteolytic action complicates the picture The clotting times were then measured in silicone coated tubes (in glass they were too short to be accurately determinable) and it

was found that the STI had increased the clotting time substantially, whereas the trypsin shortened it greatly, probably due to the damaging of blood constituents by the enzyme. Thus we observed another instance in which when the zeta potential was increased moderately the tendency of the blood to clot was decreased moderately.

TABLE XVIII

ZETA POTENTIALS WITH SOLUTIONS OF CRYSTALLINE SOYBEAN TRYPSIN INHIBITOR, CRYSTALLINE TRYPSIN AND THEIR MIXTURES WITH HUMAN FIBRINOGEN FRACTION I

Additive to 0.001 M Phosphate buffer mg./100 ml.	pH	Streaming Potential mv./cm. Hg	Specific Conductance mhos/cm. x 10^4	Zeta Potential mv
5 mg. Fibrinogen	7.1	0.83	2.34	—19
2.5 mg. STI	7.0	4.17	2.22	—89
7.5 mg. STI	7.1	5.10	2.02	—99
2.5 mg. STI and 5 mg. Fibrinogen	7.1	1.31	2.39	—32
10 mg. Trypsin	7.2	0.35	2.84	—10
10 mg. Trypsin and 5 mg. Fibrinogen	7.0	0.93	3.05	—28

TABLE XIX

THE EFFECT OF SOYBEAN TRYPSIN INHIBITOR AND TRYPSIN OF THE CLOTTING TIME OF WHOLE BLOOD

Additive to 2.5 ml. whole blood	Concentration of Additive	Clotting time
0.5 ml. 0.01% STI in isotonic saline	0.0016%	29.5 min.
0.5 ml. 0.01% Trypsin in isotonic saline	0.0016%	less than 4 min.
0.5 ml. isotonic saline	0	15.7 min.

SUMMARY

No striking difference in zeta potentials appears when a very dilute solution is streamed through vitreous silica and silicone-coated capillary tubes. Apparently the same protein (s) coats both of these surfaces equally well, and the zeta potential does not offer a clear explanation of the different clotting times of blood in glass

and silicone However, it is possible that the pH of 6 9 used here might be in the critical region where the surface coatings become identical for the work of Meyer and Meyer on the electric mobilities of quartz and collodion particles in human serum indicate that the two types of particles take on different protein coatings at higher pH values, but that their mobilities become identical under conditions of lower pH Work is now in progress to determine whether a higher pH will give significant differences in the zeta potentials of glass and silicone surfaces Another possibility is that protein may be adsorbed or bound to the wall by a rather different action on the two surfaces If it should turn out for instance that the protein coating could be readily removed by washing from the silicone surface and yet be apparently denatured on the glass surface it is possible that the difference in the effect of the surfaces on clotting rates might be explained in that way

In view of the fact that whole blood gave a lower zeta potential than the plasma it was felt that this difference if significant, must be due to red cells or to a product formed by lysis of them probably hemoglobin The zeta potential of very dilute solutions of red cells, which had been washed free of plasma with physiological saline was −11 mv which is compatible with the isoelectric point of 6 78 for hemoglobin It appears that the capillary surface is influenced by the factor in whole blood resulting in lower zeta potentials (−6 mv) and that this factor disappears when the washed red cells and plasma are measured separately

At present it is not known definitely which of the proteins of the plasma coat the vessel to determine the zeta potential However since Fraction III of bovine plasma which is 88 percent beta globulin gives a zeta potential of −14 mv and bovine fibrinogen produces a zeta potential of − 16 mv it is quite likely that either one or both of these compounds are the essential surface coating compounds since the zeta potential of native human plasma is −14 mv The synthetic mixture of fibrinogen and crystalline albumin likewise indicate that fibrinogen has a greater affinity for capillary wall than does albumin

Perhaps the most striking feature of this work is the manner in which heparin affects the zeta potential of the components involved in the blood clotting systems In general heparin causes the zeta potentials of very dilute solutions of whole blood plasma serum gamma globulin beta globulin fibrin thrombin and thromboplastin all to become more negative It has little or no effect on bovine crystalline albumin or bovine Fraction IV which is mainly albumin

These increases in zeta potential are considered not to be due to a change in ionic strength brought about by the addition of heparin but more to the formation of a complex of the plasma component with heparin as suggested by Chargaff[9] Dr Jaques[12] has shown how the formation of such complexes obeys the mass law and has measured equilibrium constants for a number of such complexes Fischer[13] has shown that heparin shifts the isoelectric point of some proteins to the acid side of the normal isoelectric pH This shift would cause the zeta potential to become more negative but the reason for the shift is probably due to the formation of a heparin complex If the heparin were desorbing the protein from the surface one would expect a high negative potential corresponding to that of heparin alone however the zeta potentials were intermediate between those of the plasma or plasma component and the heparin with the exception of the crystalline albumin Additional evidence for the heparin complex is furnished by the precipitate formed when protamine and heparin are mixed in suitable concentrations[14][12] which parallels the change of the positive zeta potential for protamine to a negative one after the addition of heparin

Of interest also is the similar action of two other anticoagulants namely paritol and "phosphatide inhibitor" which likewise cause the zeta potential of fibrinogen to become more negative presumably by complex formation However 1 5-pentanediol which Dr Ferry[15] found to inhibit the action of thrombin on fibrinogen has no effect on the zeta potential of blood which is not surprising in view of its nonionic nature

REPULSIVE FORCES ARISING FROM THE ZETA POTENTIAL

Since it appears probable that agents important to the process of clotting do produce changes in the zeta potential, a few words on the mechanism by which repulsive forces arise when two charged surfaces approach seem desirable In Figure 27 in the region L is shown the variation of the potential (solid line) and of the ion concentrations (dashed line) for a plain surface of a solid slab S in contact with an infinite layer of solution The potential here assumed to be negative falls off toward zero with increasing x and the ion concentrations $n+$ and $n-$ approach the ion concentrations of the bulk of the solution It will be observed that concentration of positive ions $n+$ is considerably increased near the surface because of the attraction of the negative potential while the concentration of negative ions $n-$ is somewhat decreased

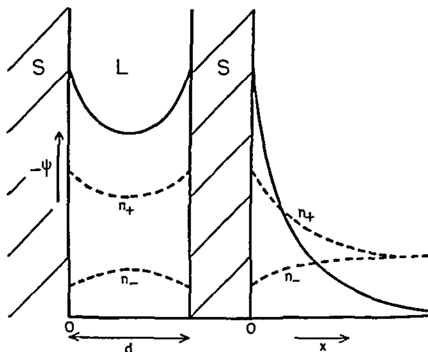


FIGURE 27

Comparison of the potentials and ion concentrations (n+ and n−) of double layers L is the diagram of two double layers interacting as a result of the approach of two slabs S L is the double layer at the surface of a solid in contact with an infinite solution as in Figure 1

because of repulsion Now suppose that two slabs S have approached to a distance of d with the solution L between them The solution part of the double layer which extended a considerable distance in L, is now compressed between the two slabs S and an interaction results, giving rise to a repulsive force The electric potential is now described by the solid curve in L and it no longer reaches zero, although it is here assumed to have the same value at the surfaces as in L (An alternative assumption leading to a *different* picture and larger repulsive forces *is* that of constant charge density on S) As a result of this potential curve the ion concentrations n+ and n− are quite different and the total ion concentration between the plates is higher than in the bulk solution Consequently there is a difference in osmotic pressure between the region L and the bulk of the solution tending to draw water into L and push apart the slabs S It is this force which is believed to prevent contact between colloidal particles and applies to a par

ticle approaching a wall, as well as two particles in solution. The particles must be as large as a big protein molecule for this force to become appreciable A quantitative discussion of this theory has been given by Langmuir and others[6][7] While it is necessary to idealize such systems to make possible calculations of these forces it is probable that the conclusions drawn from such considerations are not essentially in error as regards the actual systems provided care is exercised in extending them

On the basis of these results it may not be inappropriate to indulge in speculation about the nature of the clotting process and as Dr Best stated last year it may be very wise to ask the question why does blood not clot? It seems not unreasonable to suppose that blood has within it all the necessary substances to make possible clotting but that there is something which prevents this process from starting Is it possible that the "something" is the high zeta potential of the normal healthy blood vessel surface? It has been shown in this work that those substances which act as anticoagulants in general bring about an increase in the zeta potential of the container wall This is of course proceeding on the assumption that the clot initiates at the solid surface One of the first questions to ask is whether the forces resulting from the zeta potential are of the order of magnitude to prevent fibrinogen molecules from approaching the solid surface and forming bonds with successive molecules then growing out and extending the surface into the solution. While this system is too ill-defined to permit exact analysis such forces can be readily calculated for two other rather similar systems In order that the approach of a charged fibrinogen molecule to the solid surface be discouraged the repulsive force must be of the order of the thermal energy (kT) of the molecules of the solution which is 4 x 10^{-1} ergs/molecule at 25 C Assuming an end on approach it is reasonable to believe that surfaces of the order of 1000 A^2 are being brought together and in Figure 28 is shown the ratio of the repulsive energy per 1000 A^2 to the thermal energy (V/kT) for two plane surfaces approaching with zeta potentials in the range actually observed It is found that at a zeta potential of somewhat less than 25 mv the repulsive energy per thousand square angstroms of the double layer interaction is just equal to the thermal energy of a molecule (1 e V/kT = 1) Actually this picture is only a very rough approximation of the desired one and gives slightly higher values of the energy than would a more exact calculation Since the detailed knowledge of the surface of a fibrinogen molecule is lacking it seems futile to devise a more

elaborate picture It is nevertheless apparent that the electric double layer interaction gives rise to a repulsive force of such magnitude that the zeta potential may be a critical factor in preventing fibrinogen molecules from making contact (The preceding calculations are based on the tables of Verwey and Overbeek[7] p 66 et seq)

In order to find out whether the charge density needed to cause the observed zeta potential is of a reasonable magnitude, the number of charges per 1000 A^2 was calculated with the Poisson Boltzmann equation for several zeta potentials in a 0 15 M solution of sodium chloride and found to be about one charge per 1000 A^2 for zeta potentials around 18 mv as seen in Table XX. This means that the area corresponding to the end of a fibrinogen molecule would have about one negative charge assuming the fibrinogen surface has the same potential as the wall surface This seems to be a reasonable value because the fibrinogen molecule would need at least one charge on the end for the zeta potential to play any part at all in the process and there might very well be several charges Thus one charge on the end is a minimum and the ratio V/kT was calculated for the end of a fibrinogen molecule with a single charge approaching a plane surface The dashed lines in Figure 28 show this ratio for zeta potentials of 25 and 50 mv neglecting the effect of the single charge on the electric double layer It is evident that this picture leads to essentially the same conclusion as the former but with somewhat lower V/kT values and it may be safely con

TABLE XX

SURFACE CHARGE DENSITIES NECESSARY TO GIVE RISE TO VARIOUS ZETA POTENTIALS

Zeta potential	Charges/sq angstrom	Charges/fibrinogen molecule*
75 millivolts	6 0 $\times 10^{-3}$	460
50	3 4	260
25	1 5	115
18	1 0	77
15	0 84	65
12 5	0 71	55

* Area of fibrinogen molecule is calculated as 77 000 sq angstroms assuming it is 700 A long and 35 A in diameter

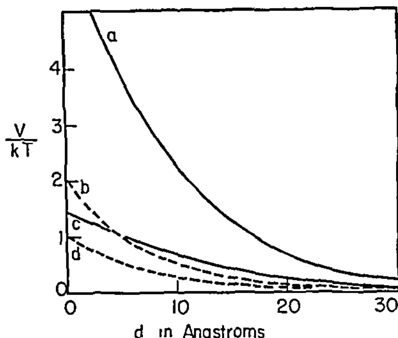


FIGURE 28

Ratio of the repulsive force (arising from double layer interaction) and the thermal energy of particles as a function of distance between particles The curves a and c are for two approaching surfaces 1000 A^2 in area, of zeta potentials 50 and 25 millivolts respectively The curves b and d are for single charges approaching a plane surface of 50 and 25 millivolts zeta potential respectively The solution is assumed to be 0 15N NaCl

cluded that the true picture whatever it may be would not differ greatly from either of these

The meaning of these deductions may be made clearer by an analogy the thermal energy of a fibrinogen molecule may be likened to a ball rolling about on a flat surface to the right of the curve lines in Figure 28 and this ball is then rolled in the direction of the wall represented by the ordinate on the left If the energy of the ball is great enough it will cause the ball to roll all the way up the curve to the ordinate and make contact The average thermal energy of the fibrinogen molecules is such that the ball has only enough on the average roll to carry it up the curve to a V/kT level equal to one which would permit contact on the curve d but not on a b or c Actually of course a certain fraction of the molecules will always have much greater energy so it would be more precise

to say that the probability for contact between the molecule and the wall is decreased for higher zeta potentials The large mass of the fibrinogen molecules tends to make the distribution of energies about kT relatively narrow, however

The significance of all these considerations is simply that the repulsive force arising from the zeta potential is of the proper order of magnitude to be critical for this system These same considerations would of course apply to two fibrinogen molecules coming into contact in solution but have not been discussed in that connection since the experiments described give information only about the wall

One or two other things might be mentioned in support of this general idea If the temperature drops V/kT increases somewhat in value at constant zeta potential and this means that the probability of approach by a fibrinogen molecule to the wall becomes smaller This may be a coincidence but if blood is chilled the tendency to clot does diminish

Another thing which has been found is that if the electrolyte concentration in blood or in fibrinogen solutions is increased the tendency to clot is diminished (Shulman and Ferry[16] W F H M Mommaerts[17]) I have not read that Dr Ferry has been able to stop clotting by adding a great deal of electrolyte to the fibrinogen solution but when he does add the electrolyte in higher concentrations it is found that the more transparent type of clot is formed With other added substances it is found that as the clot becomes more and more transparent eventually it reaches the point where it does not form at all As electrolyte is added to a solution of fibrinogen as with most proteins the number of charges and the zeta potential of the protein surface is increased because it brings about more ionization of polar groups Consequently the repulsive forces operating between fibrinogen molecules or between fibrinogen molecule and solid surfaces of clot already formed on the wall of the containing vessel would be strengthened and V/kT becomes larger If it becomes large enough there is presumably no clot formation

We venture the hope that these experiments and discussions will invite consideration of the zeta potential as a factor in blood coagulation

LIST OF MATERIALS AND THEIR SOURCES

The materials used in these experiments and their sources are

as follows

Potassium Phosphate Solution This solution was prepared by mixing 50 00 ml of 0 1 M KOH with 50 00 ml of 0 1 M KH_2PO_4 Then 10 00 ml of this solution was diluted to 1000 ml. with distilled water (the K^+ ion concentration was 0 001M)

Blood Plasma and Serum Supplied by normal humans

Heparin Supplied by Roche Organon Inc Nutley N J (1 cc = 10 mg)

Bovine Plasma Fractions Supplied by Armour and Company Chicago Ill A brief description of these samples follows

Fraction I — bovine fibrinogen (40% sodium citrate 42-48% clottable protein)

Fraction II — 100% gamma globulin

Fraction III 1 (c155) — 88% beta globulin 10% alpha globulin 2% albumin

Fraction IV (c143) — 20% beta globulin 25% alpha globulin 55% albumin

Crystalline albumin — Armour crystalline bovine albumin

Pantol Supplied by Wyeth Inc Philadelphia Pa (1 cc = 10 mg)

"Phosphatide Inhibitor" Supplied by Dr R S Overman of these laboratories

1 5 Pentanediol Supplied by E I du Pont de Nemours and Co Electrochemicals Department Wilmington Del.

Thrombin Supplied by Parke Davis and Co Detroit Michigan

Thromboplastin Dr R S Overman of these laboratories kindly supplied this material which was prepared by adding 2 ml of distilled water to 50 mg of rabbit lung thromboplastin and incubating at 37° C for 10 minutes The suspension was then centrifuged and the supernatant liquid was added to 200 ml of phosphate solution (The concentration was approximately 5 mg of thromboplastin per 100 ml of phosphate solution)

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DISCUSSION

Edsall Dr Ferry brought up one point last year which I do not think you discussed today It concerns the stability of these potentials Suppose you pass a fibrinogen solution through the capillary

and then pass salt solution through after that is there a return to the same potential you had without the fibrinogen?

Wood We have in mind conducting experiments of that sort, particularly with the idea of comparing glass and silicone surfaces I would be willing to say that that removal of fibrinogen from the surface of the capillary requires a more drastic agent than the dilute salt solution I believe a cleaning agent such as soap would remove it.

Edsall I should expect fibrinogen to stick pretty tightly to the surface in the presence of ordinary salt

Another thing I was wondering about was the effect of the temperature on the potential As the temperature is lowered the ratio V/kT increases This gives the direct temperature effect but does not the dielectric constant (D) also come into the expression for the potential? That would increase with decrease of temperature the resulting effect would be comparable to the variation in T itself and would work in the opposite direction The product DT does not change much for water between 0 and 50 C

Wood The dielectric constant changes somewhat and of course the viscosity does too but it has been shown that the temperature effect on the zeta potential is not very large As the dielectric constant decreases evidently there are compensating effects of some sort A temperature difference of even 25 degrees with salt solutions in contact with silica does not give a very pronounced change in the zeta potential Of course the temperature effect in V/kT is by no means sufficient to account for the vastly different rates of clotting of blood at 37 degrees and at zero degrees You can only account for this by assuming that, in order for blood to clot, only certain fractions of very high energy fibrinogen molecules are effective in the clotting reaction By dropping the temperature by 37 you may diminish that fraction severalfold but this I think, is a rather tentative argument

Edsall As you mentioned the very low salt concentrations you used with the whole blood would presumably hemolyze the red cells I wonder if you might want to study a system with the red cells intact I suppose you could increase the molar concentration of solute by adding a nonelectrolyte and still keep your salt concentration and hence the conductivity very low I don t know whether that would have any particular advantage with the very small amounts of red cells present in the very diluted blood with which you work.

Wood It was the conclusion of Dr Horan and I certainly agreed with him that since the plasma and the serum both gave the same effect, and since the fibrinogen alone gave the same effect as the blood with the hemolyzed cells presumably the fibrinogen was the one thing they had in common and therefore we believed we could neglect hemolysis This should be taken into account and we hope to avoid it altogether in the near future by being able to use whole blood in the capillaries

Ferry When you add a little fibrongen to 0 001 M potassium chloride, how does the protein stay in solution at such a low salt concentration?

Wood There is not very much protein for one thing

Ferry True!

Wood However it seems likely that it does not stay in solution very well. I would presume it more or less came out on the walls of the container which is the reason it had such a drastic effect on the zeta potential

Ferry It seems perhaps an unnecessary complication to increase the conductivity and the protein concentration at the same time One could have a protein solution of a given salt concentration prepared by dialysis and dilute that with another salt solution thereby keeping the conductivity constant throughout

Wood That has been tried The attempt was made to get a relatively concentrated solution of fibrinogen compared with what we used and we had difficulty with the fibrinogen precipitating right out and gelling in a way that we could not always put it back into solution

Ferry In other words if the fibrinogen concentration is at all moderately high then you cannot work at a low salt concentration to fulfill your electrical requirements?

Wood Yes

Ferry When you use a mixture of heparin and fibrinogen the zeta potential is intermediate between the zeta potential of the two Is that correct?

Wood Yes

Ferry Do you think a complex is adsorbed? Or have we a situation where the two molecules are competing individually for space on the surface?

Wood Those would be difficult questions to answer My guess would be that there is a layer of fibrinogen with the heparin going on top I doubt very much that the fibrinogen would come off again The fact that it does give you an intermediate potential indicates

it is not merely a filling up of empty spaces I think the surface becomes well coated with fibrongen

Ferry Do you know of any evidence of combination in solution from electrophoretic studies of heparin and fibrinogen?

Wood No that would be very interesting evidence to have I think to see whether the fibrinogen in solution was given a charge by the heparin

Mann In a report by W Rashkind R Ireland and W Platt (Project NM 007039 Report #20 March 18 1949 Naval Medical Research Institute National Naval Medical Center Bethesda Mary land) the statement is made that when heparin is added to whole plasma and ethanol fractionation is done the heparin comes down with the fibrinogen

Ferry There is one question about this work that worries me Do we think coagulation is a heterogeneous or homogeneous re action in the language of the physical chemist? It seems to me that the answer depends a lot on whether we are talking about whole blood or fibrinogen reacting with thrombin Isn t it true that the effects of surfaces described by Dr Barker last year were largely concerned with the effect on platelets or at any rate with far more complicated systems than just with fibrinogen and thrombin? If we have fibrinogen and thrombin reacting alone do we think that the reaction is markedly affected by surfaces? I personally don t, except in certain highly special marginal circumstances It seems to me just from observation that this reaction takes place uniformly throughout the body of the solution There are circumstances how ever where perhaps the surfaces are particularly important. For example around pH 5 we have observed that the clotting is very slow and that it does definitely begin at the surface and work in also possibly it begins when the concentration of thrombin is extremely low

Wood I would certainly agree with you I would not for one minute contend that your fibrinogen thrombin systems were af fected by the wall Certainly that would be noticeable Perhaps the point of view (which may be a very naive one) that needs to be adopted here is in effect that blood is a system which is essentially on the verge of clotting all the time and the question really is as Dr Best said last year why does it not clot rather than why does it clot Thus a relatively minor disturbance such as a surface effect may initiate the process This is the rather naive hypothesis on which we have been proceeding in these studies If we studied a

system which could not clot at all, we might not be able to discover that by running only a zeta potential experiment

Edsall In whole blood isn t the influence of the surface likely to be determined by its action in promoting the formation of thrombin from its precursors by some of the complicated mechanisms that have been referred to earlier today and will be taken up again tomorrow? In that case the rate of thrombin production may be the rate controlling factor for the speed of the whole process whereas in the kind of purified system of fibrinogen and thrombin that Dr Ferry and I have studied the thrombin is already present in sufficient amount throughout the body of the solution In this artificial purified system the reaction can start anywhere through out the whole solution, and there is no special reason to suppose that the interface is a particularly active region

Wood You have a solution there which in effect it is impossible to keep from clotting whereas presumably the blood is one which is just on the verge of clotting but which needs something to start it. I am not sure how we can fit in the idea that the interaction of the thrombin precursor with the wall is the important one here Perhaps it is really a matter of whether the thrombin or its precursor can approach the wall to undergo change of some sort We should think more about this possibility

Edsall The point Dr Ferry raised about the fibrinogen being insoluble in your system made me wonder if there were some suit able solvents which would not be too electrically conducting There are some globulins like beta lactoglobulin for which Dr Ferry used glycine as the solvent in order to get enough into solution to measure the dipole moment of the protein without raising the con ductivity of the solution very much I am afraid glycine is not so effective on fibrinogen as it is on some other proteins We know from work in our laboratory by Dr W B Dandliker, that in high concentrations glycine is actually a precipitant for fibrinogen That is so at concentrations around one molar although at considerably lower concentrations glycine increases the solubility of fibrinogen

Another thing that might be tried is to dissolve the fibrinogen in salt solution enough to keep it in solution and run it through the capillary then wash out again with fairly concentrated salt solution to remove any soluble fibrinogen and then pass through a very dilute salt solution In that case you would have fibrinogen deposit ing from a true solution rather than from a suspended precipitate

Wood We are proposing for the near future to give a precoat ing in effect of the protein and then stream various solutions through the tube I think that is a very good idea

Wright Any further comments or questions?

Flynn I had a question I wanted to ask Dr Ferry last year and that is has anybody studied the effect of free radicals such as benzoyl peroxide on the polymerization of fibrinogen?

Ferry Not as far as I know

Flynn Are there many analogies to polymerization of fibrinogen with other better known polymerizations?

Ferry I don t know of any discussion of that possibility

Edsall Most polymerizations can be classed as either addition or condensation polymerizations Addition polymerizations in general proceed by a chain reaction mechanism It seems very unlikely that the fibrinogen polymerization goes that way The analogy would probably be closer with condensation polymerization Dr Ferry can give a better opinion than I as to how close it really is

Ferry In addition polymerization the free radical is propagated so to speak from one end of the monomer to the other It is difficult to see how it could be propagated all the way along the fibrinogen molecule 700 angstrom units from one end to the other

Wright It might be noted that one of the most encouraging aspects of this study as it has progressed in the last year has been that thus far without exception those substances tested which might be termed as anticoagulants have moved the charges more in the negative direction whereas those substances which have to do with clotting have moved the charges in the opposite direction

Jaques With regard to the effect of temperature might I suggest to Dr Wood that the high temperature coefficient of an enzyme reaction probably explains the temperature effects on the clotting system does it not? I gathered that some members of the group objected to Dr Wood s experiments since he has had to use such low concentrations of plasma fibrinogen etc It is the same problem that we have had in our polarographic studies These physical methods do not allow the use of solutions of electrolytes as concentrated as those of plasma It is therefore necessary to use the very diluted systems The question was raised with regard to using heparin in the concentration of 5 mg percent I think what one is confronted with is this if one uses heparin in a dilution equivalent to the dilution of plasma and since presumably these reactions follow the mass law one ends with a system where the heparin protein compounds are completely dissociated In such a system one cannot expect any effect from the heparin Therefore the only thing to be done is to increase the concentration of heparin in the system (or of any other factor that one is studying) One

cannot use the concentrations present in normal clotting studies since the reagent must be added at higher concentrations to take advantage of the mass law One assumes then that the final system is one with a compound similar to that in the original plasma I don t know any other way out of this difficulty It is the problem of trying to set up something comparable to what is originally in plasma under completely different conditions

With regard to the results with protamine and heparin, they are very interesting to me in terms of seeing the combination of protamine and heparin Some years ago using the neutralization of the anticoagulant activity of protamine and heparin, I obtained on this reaction results comparable to those with the measurements reported The picture again is that you have combination of these two compounds according to the mass law with some dissociation It is only when you get an excess of one of the reactants that you end up with the other reactant completely combined in the compound It is also particularly interesting that the actual ratio of heparin to protamine obtained by Dr Wood came out to be the same as the ratio obtained by us (Jaques L B Charles A F and Best C H Administration of heparin *Acta med Scandinav* 90, 190 (1938)) because with different preparations of protamine the ratio varies Your particular preparation by this test happened to come out with the same answer as the type of preparation that we used in our tests

Apropos of observations on the effect of protamine on surfaces attention should be drawn to observations of Danielli (Danielli J F Capillary permeability and oedema in perfused frog *J Physiol* 98, 109 (1940)) since this may easily become lost in the literature Danielli measured the rate of development of edema in the frog hindleg preparation The addition of platelets to the perfusion fluid markedly reduced edema formation Traces of a protamine clupein reduced both rate of flow and edema formation when added to a perfusion fluid containing gum acacia but increased edema formation when the perfusion was dilute serum

Finally, (I say this in deference to Dr Garrott Allen) since there has been considerable mention of the effect of surfaces on clotting particularly for whole blood and also of the idea of clotting starting from the surface and coming in to the blood I believe someone should recall at this time that much of this was established by Lister In particular one of his experiments demonstrated this last point very nicely (Lister Baron Joseph *Collected Papers* I 125-W Oxford Clarendon Press 1909)

Brinkhous Dr Wood would you expect comparable electrostatic forces at an air liquid interface?

Wood Ordinarily you would not expect such comparable forces at an air liquid interface However it is well known that proteins denature at these interfaces so in protein solutions the answer is you might possibly expect such effects because of the peculiar denaturation of proteins

Ferry I was particularly interested in Dr Woods calculation of the electrostatic energy which has to be overcome to bring a molecule head on against the wall because I recently had occasion to calculate the energy which has to be overcome to bring two fibrinogen molecules end to end together I used an entirely different model from Dr Woods supposing that the fibrinogen molecules are both infinitely thin each having only the one dimension of length and each having a charge which is uniformly distributed along its length The two just touch at one point In this case taking the net charge of fibrinogen at about pH 7 and using an expression for the activity coefficient of a long thin rod in accordance with this model derived by Dr Kirkwood of the California Institute of Technology and taking the salt concentration as 0 15 molar the required energy is about 200 calories per mole – about one third of kT I thought we were in disagreement in discussing this privately but we are not far from agreement

Allen Dr Brinkhous question may have answered the one I had in mind Is there reason to suspect that the changes in the charge of the type you are measuring would not be affected by surface tension?

Wood Certainly the energy of the electric double layer would be one of the factors determining the surface tension Whether that would play an important part at the air surface of blood I really hesitate to guess but it might very well play a substantial part if there was a fairly high degree of ionization of the surface layer

Allen We have been disturbed by the fact that surface tension may be altered in a number of diseases and yet the clotting mechanism so far as it can be measured appears normal If surface tension is important in coagulation and abnormal bleeding it would seem that other factors must also be present

Wood In the surface tension of a liquid the main thing which you are measuring is a quantity which is related to the forces tending to hold the molecules right on the surface together The molecules which will concentrate on the surface of a plasma solution would be pretty difficult to guess but I should think that you

would have on such a surface a denatured layer of protein which would be essentially hydrocarbon in nature, so that the surface which you have would then be more similar to a hydrocarbon surface than to anything else When you measure the surface tension you are really measuring something which is related to the forces holding the hydrocarbon chains together and any electrical effects would be secondary in importance I don t think you would notice any pronounced changes in the electrical effects by the surface tension measurements although you would certainly notice other things

Alexander May I mention some experiments we did years ago with certain surface active agents? Aerosol T and zephiran in extremely minute concentration had a tremendous effect in retarding coagulation of whole blood These are just isolated observations which we confirmed time and again We could not quite interpret the results We were aiming for something that would accelerate clotting in the hope that by adding the detergent we would get more surface activity Contrariwise we found that they were anticoagulants

Wood Dr Sheppard predicted that they would be anticoagulant in activity, or at least guessed they might be We thought they would be and we thought also they should coat the wall surface and give a highly negative charge to it However we have not as yet measured any zeta potentials of these materials We have on order and plan to measure a number of different detergents

THE RATE OF COAGULATION OF BLOOD AND PLASMA IN CONTACT WITH GLASS, SILICONE, AND SIMILAR SURFACES

L M TOCANTINS R H HOLBURN
R T CARROLL and J W STOKER*

Department of Medicine Jefferson Medical College

In connection with the interesting work presented by Dr Wood it may be mentioned that the clot accelerating effect of glass and similar contacting surfaces is mediated through the phases of coagulation preceding the formation of thrombin Once thrombin is formed the type of surface matters little insofar as the rate of clotting is concerned The closer to the stage of formation of thrombin that the blood or plasma is allowed to progress the less difference there will be between clotting times on glass and silicone Over a wide range of concentrations thrombin clots fibrinogen with equal rapidity in glass or silicone tubes

For a clear demonstration of the full effect of contacting surfaces on blood coagulation

a) The blood must be collected swiftly and without admixture with tissue juices

b) It must be kept out of contact with glass until just before testing

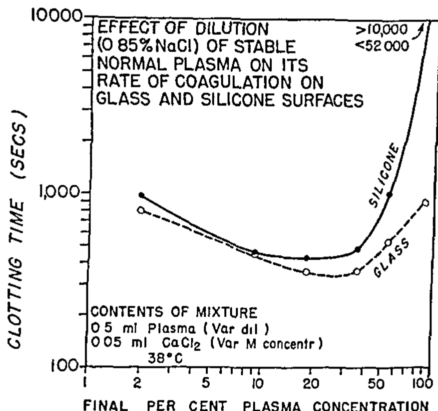
c) It must not be unduly diluted during the addition of decalcifying anticoagulants or in the preparation of the actual clotting mixture The nearer the blood or plasma concentrations in the clotting mixture are to that of the circulating blood, the greater the difference between the rate of coagulation in glass and surfaces like paraffin collodion or silicone(1)

d) The blood or plasma must have at the start an adequate content of stabilizing inhibitors (e g antithromboplastin) Whether or not the blood retains the full effective action of these inhibitors depends largely of course on the treatment the blood receives during its collection, storage and testing

For the present we shall limit ourselves to the presentation of the evidence relating to the extent *plasma concentration* influences the rate of coagulation in glass and silicone surfaces As is well known hemophilic plasma has a prolonged clotting time even in

* Aided by a grant from the U S Public Health Service

glass tubes The differences between the rate of clotting of this type of plasma in glass, silicone or collodion are greater than those observed with normal plasma By studying a plasma of great stability like the hemophilic, with normal plasma we may best appraise the influence of contacting surfaces on coagulation



(Presentation Rate of Coagulation of Blood and Plasma in Contact with Glass Silicone and Other Surfaces L M Tocantins 1950 Macy Transactions p 106-109)

FIGURE 29

The plasmas were handled by the silicone technique up to the point when the clotting mixtures were made in each tube

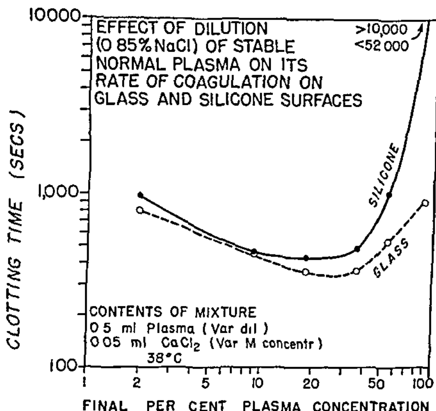
Figure 29 illustrates what happens to the rate of coagulation of stable normal plasma in glass or silicone tubes before and after dilution with 0 85 percent NaCl In this and subsequent charts depicting serial changes in plasma concentration the plasma was diluted in the tube where the clotting time was measured by adding the correct amount of saline solution to the plasma and following it with the optimal amount of CaCl For example in order to

obtain a concentration of 89 percent 0 5 ml of citrated plasma (with an original concentration of 98 percent after allowing for the citrate solution) is added to 0 05 ml of 0 2 M CaCl A plasma concentration of 30 percent was obtained by arranging the following mixture 0 17 ml of plasma 0 21 ml of 0 85 percent NaCl 0 17 ml of 0 02 M CaCl The measurements of the plasma were made with siliconized serological pipettes In order to obtain a concentration of 89 percent in the first clotting mixture it is necessary to start with a plasma of a concentration of 98 percent This is done by collecting 10 ml. of blood into siliconized syringes containing 0 1 ml of 38 percent trisodium citrate With a hematocrit of 40 the resulting plasma is diluted 59/60 by the citrate equivalent to a plasma concentration of 98 percent The plasmas were unless otherwise stated separated from the blood cells by one hour centrifugation at 3 000 r p m Only the upper three fourths of the plasma was removed and placed in silicone tubes

At a concentration of 89 percent stable normal plasma in silicone tubes has a greatly prolonged clotting time (Figure 29) At 50 percent concentration normal plasma clots in the silicone tubes at approximately the same time taken by the 89 percent plasma to clot in a glass tube Increasing dilution brings the rate of coagulation in the two types of tubes closer and closer until they are no longer significantly different when the plasma concentration is near 9 percent It may be noted that even in glass tubes dilution of stable normal plasma accelerates its coagulation so that 2 percent plasma clots slightly faster than 89 percent plasma The more stable the plasma is the more striking is the clot accelerating effect of dilution Stable normal plasma can only be obtained by an exacting technique in the collection of blood and in the separating storing and measuring of plasma

If glass in the form of fine powder is added to plasma it will accelerate its coagulation (Figure 30) The effect of glass seems more striking on normal than on hemophilic plasma Hemophilic plasma seems able to resist activation by glass to a greater degree than normal plasma The tests illustrated on Figure 30 were carried out in plasma mixtures of high concentration (78%) It can be shown that this difference in behavior toward glass between normal and hemophilic plasma can be effaced by dilution Figure 31 shows that the rate of coagulation of hemophilic and normal plasma which at 80 percent plasma concentration after activation by glass powder differs by over 4 000 seconds is about equal when the plasmas are tested at 2 percent concentration Dilution therefore

glass tubes The differences between the rate of clotting of this type of plasma in glass silicone or collodion are greater than those observed with normal plasma By studying a plasma of great stability like the hemophilic with normal plasma we may best appraise the influence of contacting surfaces on coagulation



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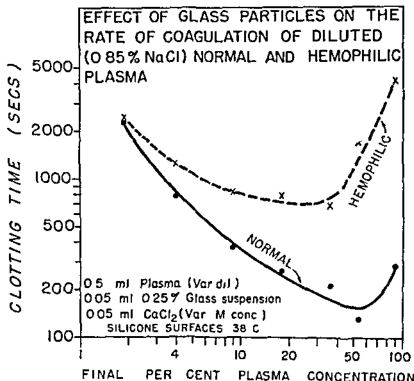


FIGURE 31

is no significant difference between the two plasmas the curves have lost their parabolic character and the clotting mixture with shortest clotting time is that at 74 percent concentration

Most workers do not realize the extent to which plasma is exposed to glass during the multiple steps required for the collection separation storing and testing of plasma Figure 33 illustrates how rapidly these changes can occur After 45 minutes of standing in a glass tube the clotting of previously stable plasma (without addition of thromboplastin) was reduced over four times

It is important also to keep in mind that it is the final concentration of the plasma in the *actual clotting mixture* that determines the response of the plasma to a given surface If stable and in high concentration the plasma will have a long clotting time in both glass or silicone surfaces the magnitude of the difference between the effect of the two surfaces depending chiefly on the final concentration of the plasma in contact with the surface Concentrations

seems to reduce the effectiveness of a factor or factors which accounts for the resistance of normal plasma and especially of hemophilic plasma to activation by glass

What happens if stable normal and hemophilic plasma are allowed to remain in contact with glass for sometime before being tested? Figure 32 portrays these changes in a condensed manner

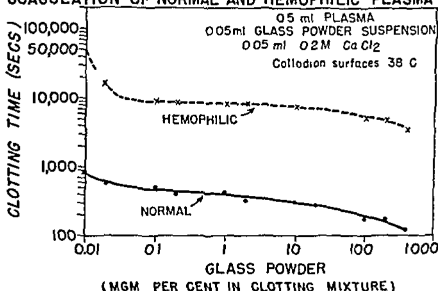


FIGURE 30

Chemically clean and washed glass powder capable of passing through a number 40 sieve (B of S) was used Concentration of plasma in each clotting mixture 78 percent

When fresh citrated normal or hemophilic plasmas previously unexposed to glass are tested in glass tubes as indicated in Figure 32 the curves for the dilution of the two plasmas assume the shape shown Even when activated by a strong thromboplastin (human brain saline extract) there *is a significant difference* between the rate of clotting of hemophilic and normal plasma when tested in concentrations of 24 percent or higher Moreover as will be discussed in greater detail later the curves have a parabolic character *After exposure to glass for two days* both normal and hemophilic plasma have shorter clotting times and their dilution curves meet at 24 percent plasma concentration After six days exposure there

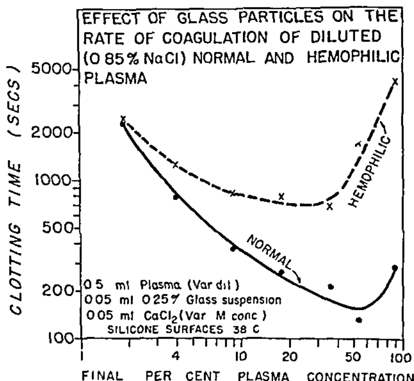


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EFFECT OF EXPOSURE TO GLASS TUBES ON THE RESPONSE OF NORMAL AND HEMOPHILIC PLASMAS TO DILUTION

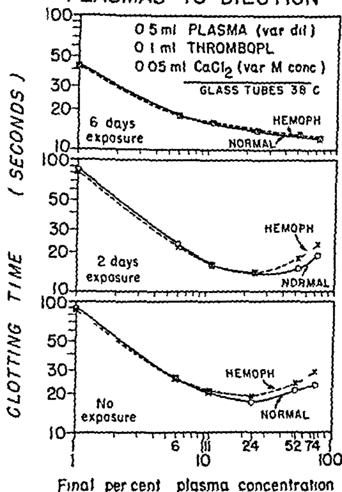


FIGURE 32

Thromboplastin *Aqueous* extract of acetone dried human brain Blood (50 ml.) aspirated into a siliconized syringe holding 1 0 ml of 19 percent trisodium citrate Dilution of plasma by citrate $= \frac{58}{60}$ (blood hematocrit 40) Dilution of plasma in first clotting mixture $= \frac{50}{65}$ Final percent plasma conc ntration $= \frac{58}{60} \times \frac{50}{65} \times 100$ or 74 percent.

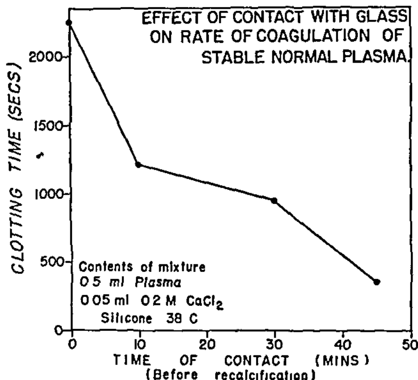


FIGURE 33

10 ml of stable normal citrated plasma collected with silicone technique placed in a glass tube for 0 10 30 and 45 minutes At these intervals 0 5 ml was trans ferred to a silicone tube and recalcified Final plasma concentration of each clotting mixture 87 percent.

of the plasma are often stated in terms of the original plasma used in the test instead of its concentration in the clotting mixture a source of much confusion This is especially evident in attempts to correlate prothrombin concentration with plasma concentration in the one stage prothrombin time method The same errors enter into experiments designed to detect antithromboplastin or anti cephalin activity The tests are done at plasma concentrations of 25 percent or below or under conditions unfavorable to the action of first phase inhibitors

Edsall Your dilutions in these experiments were made with physiological saline?

Tocantins They were made with physiological saline They have also been made with imidazole buffer pH 7 4 with acacia and with

5 percent glucose solution all with essentially the same results If the plasma is allowed to stand in contact with asbestos fibers before testing then it has a fast clotting time even in high concentration the relation between plasma dilution and clotting time may then be expressed by a straight line The curve no longer has a biphasic character

Edsall Do you know what would happen if you made the dilutions with a solution containing fibrinogen of the same concentration as that of the plasma?

Tocantins A buffered beef fibrinogen solution was tried with results almost identical to those obtained with physiological saline

REFERENCES

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THE SO CALLED PROTHROMBIN TESTS THEIR RELATIVE PRACTICAL VALUES WITH DISCUSSION AS TO WHAT WE ARE ACTUALLY MEASURING

CHARLES E BRAMBEL

Department of Clinical Chemistry Mercy Hospital and the Department of Medicine University of Maryland School of Medicine

At the risk of being repetitious there are several important ques tions which require clarification It is my hope that further discus sion by this group will materially help to remove barriers of mis understanding and lead to the formulation of a more rational pattern as personal interests give way to clear and objective think ing

Can anything new be added by discussing the question of pro thrombin tests again? There is continued need for refocussing our attention on the ever present important problem of reliable and reproducible tests for clotting activity of blood This question is reemphasized by the introduction of new anticoagulants previously discussed The following cannot be repeated too often if new anticoagulants are to be used successfully by the clinician reliable control methods are indispensable For some this problem may not present difficulties but for others there remains at least one weighty question interpretation of laboratory data from different groups of workers in various parts of the country With regard to the topic of So called Prothrombin Tests" I would like to add the following questions Does a prothrombin test exist? What is a prothrombin test? Under what conditions can plasma prothrombin be tested quantitatively or qualitatively? Why call it a prothrombin test? A good many of my remarks this morning will be aimed in the direction of attempting to stimulate discussion and thinking and refocussing our ideas Emphasis will be placed on practical phases of this topic rather than on the theoretical aspects which have been stressed during previous meetings

To attempt to answer the foregoing questions I would like to start this presentation with a problem which we encountered recently We were asked to study the blood from a leukemic patient during the terminal stages of the disease This was a case of ad vanced lymphoid leukemia and I say regretfully that we were unable

to pursue these studies to completion because the patient died The clotting properties of this patient s plasma were investigated in some detail and yielded seemingly anomalous results (Table XXI) By the one stage method undiluted plasma clotted in 40 seconds For comparison a blood specimen was selected from our daily routine dicumarol series that had a prothrombin clotting time of 40 seconds for undiluted plasma These two plasmas (one from the leukemic patient not receiving anticoagulants and one from a patient receiving dicumarol) had identical clotting times when the one stage procedure was used for prothrombin activity Considering their different origins in what manner are they alike or, in what manner are they dissimilar?

To test these plasmas having identical so called prothrombin clotting times but from different sources 50 percent dilutions with prothrombin free plasma were prepared These prothrombin free plasmas were made from normal plasmas (14 15 seconds prothrombin clotting time with one stage procedure) by treating with a series of adsorbents (barium sulfate[2] barium carbonate[3] tricalcium phosphate[4]) described as specific for removing prothrombin After addition of thromboplastin and calcium ions to the treated plasma no clotting was observed after 48 hours standing Equal quantities of the prepared plasmas (prothrombin free) were added to equal quantities of the two plasmas being studied The one stage procedure for prothrombin was done on samples of these 50 percent mixtures Surprising results ensued In one case increased activity was obtained as manifested by decreased clotting time (26 seconds) and in the other a decreased activity as manifested by a definite prolongation of the clotting time (66 seconds) Thus different results are obtained from the two plasmas when they are treated with the prothrombin free plasma The samples now reveal differences in coagulability that were not evident before

For comparison another type of diluent was used saline a 50 percent dilution of both plasmas with saline (0 85 percent) was prepared One stage prothrombin clotting times were done on these mixtures with the following results diluted leukemic plasma 64 seconds and diluted plasma from dicumarolized patient 67 seconds Under these experimental conditions to all intents and purposes identical clotting times were obtained The differences manifested when prothrombin free plasma was the diluent are now no longer apparent

The unmodified two stage procedure[3] was done on the selected plasmas and the following results were obtained leukemia plasma

TABLE XXI

TYPE PLASMA	Undiluted Plasma			50 Percent Plasma		Undiluted Plasma	
	Single-Stage Procedure	Two-Stage Procedure Unmodified		Single-Stage Procedure		Two-Stage Procedure Accelerators Added	
				Prothrombin free Plasma	Saline		
	Seconds	Percent	Thrombin Units/ml	Seconds	Seconds	Percent	Thrombin Units/ml.
LEUKEMIC	40	53	153	26	64	85	205
DICUMAROLIZED	40	39	103	66	67	46	128

TABLE XXII

CRITICAL RATIOS (C R) OF THE DIFFERENCE BETWEEN THE PERCENTAGES OF TREATED AND UNTREATED PATIENTS SHOWING COMPLICATIONS

Complications	1944 1947			1938-1947			Untreated		
	Untreated N = 2 030	Treated N = 3 304	C R	Untreated N = 9 250	Treated N = 3 304	C R	1939-1943 N = 7 2[illegible]0	1944-1947 N = 2 030	C R
Thromboembolic	0 010344	0 001210	3 9	0 008432	0 001210	6 4	0 007894	0 01344	0 99
Embolic	0 003940	0 000302	2 6	0 003027	0 000302	4 2	0 002770	0 003940	0 77
Embolic deaths	0 002463	0 000302	1 9	0 001405	0 000302	2 2	0 001109	0 002463	1 2
Total	0 016748	0 001815	4 8	0 012864	0 001815	8 2	0 011772	0 016748	1 6

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It may be in order at this time to examine the general picture of clinical progress with anticoagulants and to study by what methods control of the clotting mechanism was accomplished A review of such findings may contribute to the clarification of existing controversies

The excellent work of Doctor Barker and his associates[6] at the Mayo Clinic attests to the effectiveness of oral anticoagulants in preventing venous thrombosis or pulmonary embolism following major surgery in cases where the incidence for such complications has been established statistically Through the use of dicumarol 73 lives were saved and 211 patients did not experience thromboembolism in a group of 1 686 cases The incidence of hemorrhagic complications was minimal This impressive clinical data accompanied the use of a modified one-stage procedure for controlling the coagulation components of the blood during the administration of the drug A selected level of decreased clotting activity was satisfactorily maintained by these investigators

Our[7] contribution to this phase represents the run of the mill major surgical cases covering a period of 4 years The results were analyzed statistically because of the heterogeneity of the data For the years 1944 to 1947 comparing 2 000 untreated cases with 3 000 treated with dicumarol the critical ratio was 3 9 for thromboembolic complications 2 6 for embolic and 1 9 for embolic deaths giving us an overall ratio of 4 8 The higher the critical ratio the more significant the figures are statistically (Table XXII) Here again a modified one stage procedure was used advantageously to maintain a selected level of induced hypocoagulability for therapeutic purposes

In Table XXIII data are presented from seven groups of investigators These studies are concerned with another clinical entity namely coronary thrombosis We note that the total percentage of mortality is reduced from 26 percent to 15 percent and thromboembolism from 24 percent to 5 percent I would like to call attention at this point that the total percent figures correspond very closely to the series worked out by Dr Wright and his associates The one-stage procedure was used as a control method in these studies

In Table XXIV we have data from another clinical application [15] Over 3 000 postpartum cases have been studied Here again we observe the effectiveness of oral anticoagulants in forestalling thromboembolism Transmission of dicumarol to nursing infant across the breast barrier was not demonstrated by us [16] These

53 percent (153 thrombin units per ml) and plasma from the selected dicumarolized patient 39 percent (103 thrombin units per ml) Obviously, the differences in clotting power between them are not very striking when the unmodified two stage procedure is used

The effect of addition of accelerator factors was investigated Since purified preparations were not available at the time Ac globulin was added to these plasmas using bovine serum[3] as a source and in accordance with the directions of Seegers After the addition of Ac globulin to the two plasmas, differences in clotting potential were obtained when the two stage procedure was used leukemic plasma 85 percent (205 thrombin units per ml) and plasma from dicumarolized patient 46 percent (128 thrombin units per ml) Thus making use of some recent developments in methods for measuring coagulability of plasma a near normal value was obtained for one specimen (leukemic) and an abnormal value for the other (plasma from dicumarolized patient) Yet it is well recognized that clinically both of these patients potentially at least had bleeding tendencies one pathologically and the other induced with an anticoagulant drug All of the preceding data is summarized in Table XXI

No originality is claimed for the experimental approach used in the analysis of the foregoing cases Owren[5] has done a much better job in evaluating the weaknesses of existing procedures It was only by good fortune that we obtained the clinical material otherwise this part of the presentation would have been a direct quotation from Owren's monumental studies There is no necessity for repeating his arguments at this time

I think that the foregoing analysis reveals and re emphasizes the inadequacy of our present routine procedures for measuring the clotting power of blood Not only that but they are called by incorrect names and the definitions lack specificity The one stage prothrombin clotting time procedure does not measure prothrombin qualitatively or quantitatively The two stage procedure requires more precise focussing of definitions of conditions if it is to be used to determine the number of thrombin units in a given specimen of plasma It is sound in theory but further evolutionary development must take place before general applications can be made

Yet notwithstanding all these difficulties and objections the one stage procedure has proved invaluable as a control tool in routine clinical studies involving oral anticoagulants It appears that the principles of anticoagulant therapy have carved a permanent niche in medicine

TABLE XXIV

SUMMARY TABLE OF INCIDENCE OF VASCULAR COMPLICATIONS AND POSTPARTUM HEMORRHAGE

		Vascular Complications		Thromboembolic Deaths		Postpartum Hemorrhage	
	Number	Number	%	Number	%	Number	%
Untreated (Group I)	3,318	16	0.48	1	0.03	12	0.37
Treated (Group II)	3,284	2	0.06	0	0	17	0.51
Total	6,602	18	0.27	1	0.01	29	0.43

interpretation of the theses that the coagulationists have formulated. However, the biological aspects must not be lost in the theoretical network.

As an illustration of the adaptability of the one stage procedure I would like to review the volume of routine work involved at Mercy Hospital for the last year. Table XXV is a page from our annual report for 1949 during which period 41,363 plasmas were processed. There were 17,422 in patient and 23,941 out patient blood specimens collected. The entire number was run with the one stage procedure. In addition, 2,000 of these were tested with the two-stage method.

TABLE XXV

NUMBER BLOOD SPECIMENS TESTED—1949

Month	House Patients	Out Patients	Total
January	1,158	1,657	2,815
February	1,281	1,659	2,940
March	1,545	1,880	3,425
April	1,407	1,889	3,296
May	1,466	1,931	3,397
June	1,352	1,989	3,341
July	1,463	2,008	3,471
August	1,537	2,102	3,639
September	1,404	2,057	3,481
October	1,627	2,226	3,853
November	1,606	2,243	3,849
December	1,556	2,300	3,856
Total	17,422	23,941	41,363

TABLE XXIII

CORONARY THROMBOSIS

COMPARISON OF MORTALITY RATES AND INCIDENCE OF THROMBOEMBOLISM FOR A NUMBER OF REPORTED SERIES (CONTROL C VERSUS TREATED T CASES)

Authority	Number Cases		Mortality Percent		Thromboembolism Percent	
	C	T	C	T	C	T
Wright Marple Beck (8)	368	432	24	15	25	6
Peters Brambel (9)	86	110	25	11	15	1
Parker Barker (10)	100	50	13	10	37	4
Nichol Page (11)	0	44	0	16	0	2
Greisman Marcus (12)	100	75	35	9	21	4
Freston Taylor (13)	54	45	22	20	25	10
Zeluff Field (14)	100	80	40	25	20	5
Total	808	836	26	15	24	5

NOTE Numbers in parentheses are reference numbers

studies show that clinically and by a modified one stage procedure the anticoagulant effect was not detectable in the newborn

I have used the foregoing mass of accumulated medical data as an example of what might be called effective anticoagulation In other words in general thromboembolic complications have been successfully prevented in several clinical categories The elected control method was some adaptation of the one stage procedure for measuring clotting activity It may be true that the method leaves much to be desired from a chemical and even biochemical point of view but better ones are being sought For practical purposes one would have to go very far by way of improvement in clinical results to justify the use of more elaborate control methods

The problem of developing methods for ascertaining coagulability of blood has been approached from two points of view (a) By the coagulationists who set up heterogeneous mixtures of native (normal and pathic) and treated (adsorbed) plasma Clot activating crude tissue extracts with thromboplastic properties (relatively unknown chemical composition) are added with an inorganic ion (calcium) On the basis of the results from such experiments they attempt to reconstruct the story of blood clotting or describe the coagulation process (b) By the theoretical chemists who are interested in isolating active components in pure form and studying the mechanism of reaction It seems to me that the final responsibility rests on the ability of the pure chemists to set up models From such studies a closer approximation may be obtained for the correct

will venture an analysis of adsorbed plasma On the one hand we have a simple salt solution where one is concerned with ion effects from a small number of species and on the other a variable multiple component system

Under what circumstances is it necessary to ascertain the various components quantitatively? In academic studies extreme precision is the goal. For practical and routine clinical studies precision may be cumbersome and unnecessary It is the clotting power of blood that is related to hemorrhage or thrombosis and not the effect of any one specific component The one-stage procedure does not identify the component

The relationship of coagulability of blood to hemorrhage is still obscure Hemorrhage is not a necessary sequel to incoagulable blood Any number of other factors may be responsible Vascular integrity (capillary fragility or capillary permeability) complicates this picture and has not received sufficient emphasis Needless to say this phase of the problem is likewise extremely complex. The degree to which clotting must be inhibited to prevent thrombosis has not been universally agreed upon To determine which of the components must be affected and to what degree for satisfactory anticoagulation is still a major problem Only a small advance has been made in this direction

We processed 2 000 blood samples with the two-stage procedure Our impression with regard to this test is as follows if one were certain that the first reaction went to completion that is complete conversion of prothrombin to thrombin and arrived at an answer of so many units of thrombin per milliliter then the two-stage procedure could be used to set up standards of reference for some of the clinical problems even to what extent anticoagulation should be carried. But additional reagents have to be developed before such conditions will be possible To install the two-stage procedure as a routine method on a large scale of determinations as far as I am concerned — I am speaking only from personal experience and that limited to one years trial — does not seem feasible It is easier for me to prepare rabbit brain thromboplastin of uniform potency in large batches than to provide fibrinogen solutions of uniform potency (reactivity) and concentration accelerators from bovine serum and thrombin of uniform activity These conditions apply only where routine clinical studies are involved

I cannot see how the one stage procedure can be called "a prothrombin clotting time" Evidence has been presented here and

Table XXVI shows the in patient categories that were tested during the anticoagulation program at Mercy Hospital for 1949 A total of 2 881 hospital in patients were processed through the year The program consists of (a) routine prophylaxis in the Surgical Gynecological and Obstetrical departments and (b) therapeutic applications in the department of Medicine

If the one stage procedure is the method of choice a thromboplastic reagent of constant potency is unequivocally essential Our efforts have been directed toward obtaining a thromboplastic emulsion from rabbit brain which would be reproducible over long periods of time To date we have succeeded in preserving the reagent in emulsion form ready for use in an ordinary refrigerator for a period of six weeks without change in potency These stored preparations were checked at frequent intervals with freshly prepared suspensions and found to agree It is possible to process 4 000 blood samples over a period of one month with one batch of thromboplastin Errors in clotting time arising from the use of multiple preparations have been eliminated by preparing large batches of this reagent

Two prothrombin determinations were run routinely on each blood specimen undiluted plasma and a 12 5% saline dilution of plasma The use of physiological saline as a diluent has evoked criticism from various investigators We know the composition of 0 85% saline with a fair degree of certainty Very few investigators

TABLE XXVI

NUMBER HOSPITAL PATIENTS TESTED—1949

Month	Medical	Gynecological & Surgical	Obstetrical (Postpartum)	Total
January	4	95	112	211
February	1	133	111	245
March	6	148	128	282
April	13	119	93	225
May	7	114	104	225
June	13	139	105	257
July	5	84	134	223
August	18	126	120	264
September	17	104	128	249
October	19	100	125	244
November	16	99	127	242
December	17	84	113	214
Total	136	1 345	1 400	2 881

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16 BRAMBEL, C. E., and HUNTER, R E. Effect of dicumarol on the nursing infant. *Am J Obst and Gynec 59 1153 (1950)*
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DISCUSSION

Quick The first point I will discuss is in regard to the prothrombin curve the curve which I published in 1938 That to my mind still has a high degree of accuracy I spent literally hundreds of hours constructing it using saline primarily as the diluting agent Now with additional data available particularly from the adsorption and elution procedure to check the curve I find that the values of the points in the curve still agree

In regard to Dr Brambel s remark that when you use plasma deprothrombinized by adsorption with barium sulfate of calcium phosphate you don t know what you are dealing with I feel like shouting "Amen" Dr Brambel s studies of the two patients one having leukemia the other a hypoprothrombinemia due to dicumarol, clearly show what confusion these deprothrombinized plasmas can cause Dr Brambel asks what he is measuring in the leukemic and in the dicumarolized patient. My answer is that he is measuring free prothrombin in each case and I want to add further that were I either one of these cases and a surgeon wanted to operate on me I would jump out of bed and run just as I would were he contemplating ligating my femoral veins I think you will agree with me that either patient would probably be a candidate for hemorrhage were his vascular system injured even though the prothrombin as measured by the two-stage method is 85 percent of normal Even were I in favor of the two-stage method I would hesitate to defend its reliability as applied to these particular patients

Why are we getting these discrepancies in regard to the leukemic and the dicumarolized patients when we dilute their plasmas with deprothrombinized plasmas? I think the answer is that in addition to the prothrombin which the plasma contains there is a varying amount of prothrombinogen Since it is likely that plasma treated with barium sulfate may contain a factor which can convert pro-

elsewhere that this procedure does not measure prothrombin Other variables play an equally important role and probably a more important one which affects the test Doctor Flynn[17] pointed out last year that the one stage procedure measures only a small portion of the thrombin produced As a suggestion maybe one can call it the one stage clotting index" since no specific component is identified by it

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That generalized intravascular circulation can take place and be compatible with life when the process is not carried out too rapidly can be readily demonstrated by the slow intravenous administration of either thromboplastin or thrombin The blood of such animals can be completely defibrinated and yet the animal recovers

There exists normally a mechanism whereby fibrin can be readily removed — that of fibrinolysis Even so these facts do not explain why the absence or reduction of a certain element essential for normal coagulation should account for spontaneous hemorrhage Can it be that the normal integrity of the vascular wall is dependent upon a normal process of coagulation and that when this process is accelerated intravascular clotting occurs and when it is retarded abnormal bleeding appears?

In connection with Dr Wood s comments earlier one can but wonder whether there is a continued deposition of a fibrin protein along the vessel wall and whether or not this protects against spontaneous bleeding by creating a film such as Dr Wood demonstrated *in vitro* We have searched for such a film using Clark ear chambers and with magnification at approximately 1000 times have not been able to see it, although obviously there are many reasons why it could be overlooked by the techniques we employed

The rapid turnover of prothrombin and fibrinogen could be argued to indicate that they are consumed in the production of a fibrinogen fibrin or profibrin coating To test this hypothesis we performed the following experiments Dogs were dicumarolized and then transfused with blood to elevate their prothrombin activity As soon as the transfusion was completed the animals were heparinized for a 24 hour period This procedure we thought, would prevent the decay of prothrombin if it were being consumed in a continuous intravascular clotting process This did not occur and it would seem that some other explanation must be forthcoming to explain the relationship between the clotting mechanism of the blood and the so-called integrity of the vascular wall Perhaps diapedesis which Krogh describes as a normally occurring phenomenon proceeds to hemorrhagic proportions when the clotting mechanism is disturbed

Ferguson Apropos of Dr Allen s (*J Lab Clin Med* 34 *1579 (1949)*) revival of the old idea (Nolf P *Arch internat de physiol* 4 *165 (1906) 6 306 (1908)*) that small amounts of fibrin formation continuously occurring in relation to vascular endothelium (also leukocytes Nolf platelets Lenggenhager K *Klin Wchr* 15 *1835 (1936)*) may have certain physiological functions (? nutritive

thrombinogen to the active state, it is obvious that when a plasma is mixed with deprothrombinized plasma some prothrombinogen is converted to free prothrombin Therefore the prothrombin time of such a mixture is not a true measure of the active prothrombin in the plasma under investigation You will note that when saline is the diluting agent, there are no discrepancies Both plasmas on 50 percent dilution yield a prothrombin time of 64 to 67 seconds In that range a difference of 3 seconds is insignificant and is within the experimental error Saline dilution gives you the true value whereas dilution with deprothrombinized plasmas do not The results of the study of these two patients are very instructive and fit with the concept that I will outline later in greater detail

Brambel I would like to make just one comment about Dr Quick s remarks with regard to bleeding tendency in these two patients The leukemic patient at autopsy showed no evidence of hemorrhage anywhere The individual receiving dicumarol has not shown any hemorrhages in the last two years and has been maintained in a range of 25% to 50% of normal

Quick When the vascular system is intact an individual will not hemorrhage even though his blood is incoagulable whether it be due to a lack of thromboplastinogen as in hemophilia hypoprothrombinemia heparinization or a total absence of fibrinogen Hemorrhage occurs only when an additional factor is superimposed on the original coagulation defect This we must always bear in mind

Allen As Dr Quick has said you can give heparin clinically to normal or near normal subjects or animals in quantities sufficient to prevent blood from clotting and yet he will not bleed The same holds true but to a lesser extent for dicumarol On the other hand when spontaneous bleeding does occur from a definite clinical abnormality such as prothrombin deficiency hemophilia thrombocytopenia or too much heparin all evidence of spontaneous bleeding ceases when these particular defects are corrected or go into remission For a long time we have considered coagulation in terms of a perpetually fluid state for the circulating blood and have wondered how the circulation maintains its normally fluid character This age old point of view it seems to me is open to question It may be that circulation is not normally continuously fluid but that coagulation is continuously taking place at a restricted and limited rate The relatively short life of circulating prothrombin and fibrinogen would support this point of view The platelets also do not last long in the circulation

clinical significance *New England J Med 240, 403 (149)*) that the prothrombin time of plasma with 50 mg percent fibrinogen would be of the order of 40 seconds This is based upon experiments wherein afibrinogenemic plasma was mixed in various proportions with normal plasma and the prothrombin time of the ensuing mixtures measured As the fibrinogen concentration declined the prothrombin time went up There was no alteration in the prothrombin content but there was a variation in the fibrinogen mixture and it seemed to us evident that we were measuring by the prothrombin time not only the prothrombin content but also the fibrinogen concentration We might also cite additional observations on another child with congenital hypoprothrombinemia (Landwehr G Lang H and Alexander B Congenital hypoprothrombinemia *Am J Med 8, 255 (1950)* wherein the fibrinogen concentration was perfectly normal and the plasma had a prothrombin time of about 66 seconds There was another God given experiment where we could take hypoprothrombinemic plasma which did not have to be treated with barium sulfate since the plasma was already practically devoid of prothrombin and mix that with normal plasma No manipulation was employed other than oxalation of the plasma in the routine fashion If we could have added such observations to those listed by Dr Brambel we would have come out with entirely different prothrombin times than shown in Table XXI As a matter of fact one part of normal plasma added to nine parts of the congenitally deficient plasma gave a far shorter prothrombin time than was obtained on a mixture of one part of normal plasma with nine parts of barium sulfated plasma Accordingly it is obvious that the prothrombin time measures among other things the concentration of prothrombin the concentration of fibrinogen and the concentration of other factors We have therefore attempted to be precise by considering the prothrombin time as merely a reflection of the velocity with which a clot is formed under certain specified conditions where optimal amounts of thromboplastin and calcium are present

When saline is employed as a diluent it produces far different values than when congenital idiopathic hypoprothrombinemic plasma is used as a diluent That also emphasizes the fact that when saline is employed it dilutes out other factors or contrariwise when congenitally idiopathic hypoprothrombinemic plasma is used as a diluent it fails to dilute out other factors Be that as it may we must be limited in our interpretation All we can say is that the prothrombin time represents the interval required for the evolution

Nolf) and leading up to a hemostatic function (Nolf Allen et al), there is the very strong argument provided by rare cases of ap parently complete afibrinogenemia The English case studied by L J Witts (*J Path & Bact 54, 516 (1942)*), R G Macfarlane, H Scarborough (*Proc Roy Soc Med 38, 403 (1945)*) and J L Pin niger and F T G Prunty (*Brit J Exper Path 27, 200 (1946)*) especially emphasizes the negative capillary fragility tests non interference with capillary contraction in response to injury (Macfarlane R G *Quart J Med 10, 1 (1941)*) and nonparticipa tion of fibrin in platelet agglutination and breakdown Interesting as the speculation of a possible physiological formation of traces of fibrin intravascularly in normal blood flowing through normal ves sels may be it is an idea singularly lacking the support of concrete evidence and therefore not helpful as yet in the understanding of practical problems of coagulation and hemostasis

Alexander I would like to make a few remarks regarding what we measure by the prothrombin tests and what the limitations are of the various procedures that have been employed to date I view the situation somewhat differently from one of the speakers who stated that the ultimate responsibility is that of the physical chemist to delineate carefully and to isolate the various components and to put them together in a system where great precision can be em ployed to measure physiologic phenomena I think it is the ultimate responsibility of all of us not just that of the physical chemist to *clarify in our minds precisely what is being measured and to limit* ourselves in our considerations to the precise phenomena which we measure rather than to allocate a physiological phenomenon to a specific component

Dr Quick has stated that the data shown by Dr Brambel in Table XXI the 26 second prothrombin time obtained using barium sulfated plasma as a diluent in contrast to the 66 second value obtained on dicumarolized blood both measure prothrombin It is exceedingly difficult for me to accept that concept because of the wide discrepancy between those two figures It seems to me that it is obvious that something in addition to prothrombin is being measured We might ask ourselves what would happen if Dr Brambel among his 43 000 cases had one individual who had a fibrinogen concentration of 50 mg percent but whose prothrombin content was perfectly normal I would venture to predict on the basis of observations we have made on a child with congenital afibrinogenemia (Alexander B de Vries A Goldstein R Pro thrombin *A critique of methods for its determination and their*

can be eluted off the barium sulfate that was used to adsorb the original plasma develops the physiologic function of being able to be converted to thrombin faster as it ages That is also true of the intact oxalated plasma allowed to age Also one can obtain citrate eluates from the barium sulfate which have little prothrombin activity and still a prothrombin conversion accelerating agent can be demonstrated in the citrate eluate Dr Quick refers to that as prothombinogen Is that the term Dr Quick? A similar agent call it whatever you like can be separated from normal human serum obtained hours or days after coagulation If that is prothrombinogen I should be happy to send Dr Quick a sample of it to test its ability to be converted to prothrombin We have obtained a purified fraction of that material from serum and consider it to be a prothrombin conversion accelerator To be sure it may be a precursor of prothrombin but we might expect that during the transition of plasma to serum that prothrombinogen if it did exist as such would have been converted to natural or let us say active prothrombin and that all the prothrombin would have been consumed during the course of coagulation Hence we consider this serum factor to be an accelerator It has been separated and partially purified and we would be happy to send it to anybody to measure its ability to accelerate the conversion of prothrombin to thrombin in the course of the clotting of normal blood hemophilic blood silicone blood and heparinized blood The material is readily available

We agree entirely with Dr Mann s observation that dicumarol affects more than one component of the plasma because there is evidence that dicumarol plasma containing a 1 percent or 2 percent of normal prothrombic activity is quite different from a congenital idiopathic hypoprothrombinemic plasma containing the same order of magnitude of prothrombin by the two stage method

Wright Do you mean by that in the determination of your so called prothrombin tests you are measuring more than one component?

Alexander I am convinced that that is true

Wright In both one and two stage tests?

Alexander As to the two stage method as far as we can tell at the moment it measures the number of units of thrombin that can be evolved maximally from the given biologic specimen Whether additional factors necessary to give maximal yield will be discovered in the future as has already been demonstrated for at least one factor by Dr Seegers and his group it is impossible to say at present. But the modified two stage method of Dr Seegers and his group

of sufficient thrombin to clot a certain fibrinogen solution

We might consider, what is the effect of adsorbing plasma with $BaSO_4$? We know that barium sulfate does not alter the fibrinogen concentration to the best of our ability to measure fibrinogen We know that the antihemophilic globulin and albumin also are not adsorbed by barium sulfate We end up with a plasma which is practically devoid of prothrombic activity in the sense that it fails to clot on the addition of thromboplastin and calcium We might look at the barium sulfate for a moment and see what we can get off the barium sulfate which has been used to adsorb the normal plasma As Dr Quick pointed out and as we have reported (Alexander, B de Vries A, Goldstein R and Landwehr G A prothrombin conversion accelerator in serum *Science* 109, 545 *(1949)*) (Alexander B and Landwehr G Evolution of a prothrom bin conversion accelerator in stored human plasma and prothrombin fractions *Am J Physiol* 159, 322 *(1949)*) one can obtain quantitatively from the barium sulfate all the prothrombic activity by citrate elution I use the term prothrombic activity purposely for the sake of precision to avoid attributing it to any one single component One gets off all the prothrombic activity which when re added to plasma will in the one stage test restore the original prothrombin time

Seegers How do you know you recover the prothrombin activity quantitatively?

Alexander I don t I will elaborate further Dr Seegers We have simultaneously run two stage determinations both by the modified technique that you recommended and also by the orthodox tech nique and we have quantitatively recovered the activity in number of prothrombin units demonstrable originally in the intact plasma

Seegers How do you know that that is quantitative?

Alexander By definition a prothrombin unit is convertible to a thrombin unit and by definition of thrombin it is that entity which has the activity of clotting fibrinogen Beyond that I don t think we can go at the moment

We might also focus our attention on what else comes off the barium sulfate Dr Quick has stressed the point that if one takes the citrate eluate from barium sulfate and lets that store in that material one gets faster conversion of the prothrombin to thrombin as its ages This is in accordance with our observations (Alexander B and Landwehr G Evolution of a prothrombin con version accelerator in stored human plasma and prothrombin frac tions *Am J Physiol* 159, 322 *(1949)*) It is evident that whatever

can be eluted off the barium sulfate that was used to adsorb the original plasma develops the physiologic function of being able to be converted to thrombin faster as it ages That is also true of the intact oxalated plasma allowed to age Also one can obtain citrate eluates from the barium sulfate which have little prothrombin activity and still a prothrombin conversion accelerating agent can be demonstrated in the citrate eluate Dr Quick refers to that as prothombinogen Is that the term Dr Quick? A similar agent call it whatever you like can be separated from normal human serum obtained hours or days after coagulation If that is prothrombinogen I should be happy to send Dr Quick a sample of it to test its ability to be converted to prothrombin We have obtained a purified fraction of that material from serum and consider it to be a pro thrombin conversion accelerator To be sure it may be a precursor of prothrombin but we might expect that during the transition of plasma to serum that prothrombinogen if it did exist as such would have been converted to natural or let us say active prothrombin and that all the prothrombin would have been consumed during the course of coagulation Hence we consider this serum factor to be an accelerator It has been separated and partially purified and we would be happy to send it to anybody to measure its ability to accelerate the conversion of prothrombin to thrombin in the course of the clotting of normal blood hemophilic blood silicone blood and heparinized blood The material is readily available

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seems to me to be the most direct and reliable way of measuring the number of units of thrombin that can be evolved

We also have to consider the questions that may be asked by the clinician as well as those asked by the clinical investigator or by the physical chemist? The clinician is interested in whether a patient will or will not bleed under certain circumstances? He also wants to know whether an anticoagulant is effective or whether there is an increased tendency to coagulation? Within that frame work the one stage method is highly useful Inasmuch as the one stage method measures the velocity with which a clot will form under certain specified conditions, the clinician can be and has been satisfied If however the clinician wants to know are the hemorrhagic phenomena referable to a deficiency of prothrombin which by definition is that material which can be converted to thrombin or is the defect referable to some other deficiency then the one stage method has to be modified or amplified or give way to the two stage method because we know that fibrinogen deficiency alone would give an elevated prothrombin time Accordingly, to delineate and define the coagulation defect requires more than the orthodox one stage procedure

If the clinician asks How far am I getting with my anticoagulant? Am I interfering sufficiently with coagulation to prevent phlebothrombosis or embolic phenomena " again the one stage method will be satisfactory to tell him that he is inducing a coagulation defect of a certain magnitude If however the clinical investigator wants to know more about the interrelationship between prothrombin and other clotting factors or to study precisely a particular clotting factor or coautocatalytic factor and one can enumerate numerous problems then the one stage procedure has to be employed in conjunction with many other tests for exploration

Wright Dr Seegers I think it is about time we asked you to discuss this problem since so many of the points which have been brought up referred to your work

Seegers The paper by Dr Brambel interested me a great deal In regard to the quantitative recovery of prothrombin from barium sulfate we have never succeeded in quantitatively recovering prothrombin under the conditions discussed by Dr Alexander and I am not convinced that one can speak of quantitative recovery If you apply the two stage method to the quantitative determination of prothrombin in plasma that is one set of conditions If you apply the two stage method to the quantitative determination of isolated prothrombin that is another set of conditions and I agree

with the general trend that one cannot rely on any one procedure to come to any kind of a final conclusion whatsoever

In regard to Dr Brambel s remark referring to the final responsi bility relative to the clotting factors and their isolation that this problem will be solved by the people who do purification work I don t believe it is their final responsibility I agree that it is every one s responsibility and it brings to my mind an article that I recently read in Science I think in the September 30th issue or thereabouts "The Meaning and Limitations of Exact Science" by Max Planck I think that applies here

One more remark In all of this we have to consider one more thing and that is the possibility of an altered reactivity of pro thrombin itself That does exist We can say that this is definitely real and that it applies in our deliberations

Wright Would you like to elaborate a little on the evidence that there is an alterable activity of prothrombin?

Seegers I would like to but it would take too long The references are (Seegers W H Loomis E C and Vandenbelt J M Prepara tion of prothrombin products Isolation of prothrombin and its properties *Arch Biochem 6 85 (1945)* Seegers W H and Mc Claughry R I Production of an inactive derivative of purified prothrombin by means of purified thrombin *Proc Soc Exper Biol and Med* 72, 247 *(1949)*) We also plan to publish some work which has just been completed

Smith May I make a comment on the subject of responsibility? It seems to me that where a problem has many variables there are two general ways in which it can be approached It can be split down the middle each part still has variables but not quite as many as before By further splitting the number of variables may be reduced further This is the way many biologists approach a prob lem On the other hand the physical chemist fragments the problem to a much greater degree studying only the small pieces that con sist of two or three easily manipulated variables Obviously in this field both methods are needed The biologist needs the abstract studies of the physical chemist for proper integration of his experi ments Thus the two methods are complimentary

Seegers There is one method which adds very little to the blood clotting and it is exemplified in the data outlined in Table XXI I call this a mixing experiment because I get "mixed up" and I think everyone else does The experiment itself is not the difficulty It is the interpretations possible and the many variations of the experi ment itself Obviously it means different things to different workers

There must be a universality in the truth of the matter so that everyone can see alike and say, yes this is the way it is and this is the only way it can possibly operate It is not clear to me why we should have so many journal pages devoted to mixing experiments when a single paragraph could point out the facts and be followed by the comment that there is something to consider

Smith On the other hand don t you think that Dr Bramble s experiment is useful in showing that the problem is not simple This experiment indicates another variable and it serves a warning to all of us I for one appreciate the experiment even though the analysis has not been carried very far It is true it leaves us with an unanswered question but a question which most of us were not aware even existed

Alexander Isn t it true that the physical chemists labor under the same considerations when their data are similarly derived from mixing experiments which incidentally serve to mix me up also I can apply the same criticism to the most abstruse type of scientific data because the limitations in our perception are exceedingly high and we cannot go beyond what we can see at the moment Perhaps Dr Edsall would comment on that point

Edsall Yes I would sympathize with what you just said In all his work the biochemist is confronted with a dilemma He wants pure compounds for chemical study and he wants to maintain the integrity of the living cell and he cannot have both – at least not until after a very long struggle In order to understand the function of the living organism he must try to take it apart and however he does it he is bound to break up many of the essential organized structures that were originally there Then if he is both skillful and lucky he gets out some purified chemical compounds that may have great biological interest But he cannot stop there he tries to reconstruct the original system or something like it by putting his purified components together in various ways Almost inevitably he fails or perhaps achieves a very imperfect success Of course he goes wrong at the first try because he damaged so many of the component elements in taking them apart from the original system Then he tries again using gentler methods of separation and using every clue he can get as to what was wrong with the first procedure One has to approach these things by a long series of trials getting a little bit nearer to the undamaged state of the original system each time

I know that in some of the constituents of blood plasma one can recognize several degrees of damage to a given type of molecule

One of the lipoproteins of plasma the alpha lipoprotein which we suspect contains the heparin cofactor was first prepared in a fractionation process during the war under conditions where it was pretty badly damaged Some of the lipid was split off and the protein was obviously denatured then a modification was made in the procedure by precipitating at much lower alcohol concentration and adjusting the pH and a preparation was obtained which behaved more like a normal protein molecure (Cohn E J Strong L E Hughes W L Jr et al Preparation and properties of serum and plasma proteins IV A system for the separation into fractions of the protein and lipoprotein components of biological tissues and fluids *J Am Chem Soc 68 459 (1946)*) Now under the new procedures quite recently developed the same constituent or group of constituents is being separated with solubility properties that are quite different from what they had been even under the later procedure adopted in the war (Cohn E J Gurd F R N Surgenor D M et al A system for the separation of the components of human blood Quantitative procedures for the separation of the protein components of human plasma *J Am Chem Soc 72, 465 (1950)*) Certainly the newer preparations are at least much closer in character to the constituents present in the native plasma than were the preparations first obtained

The history of the study of nucleic acids and nucleoproteins furnishes another striking example of this type of progression The early preparations obtained by Levene and others had been drastically degraded by rough chemical treatment. Now they are being separated by much milder means and the products obtained are certainly much closer to what is present in the living cell But the early work gave indispensable leads and the later advances would not have been possible without it

Wright Dr Seegers would you like to enlarge on your statement regarding the data in Table XXI? As you have implied it means different things to different people? I think we would all be interested in your frank expression of what those figures mean or do not mean to you

Seegers First of all my interpretation of the experiment outlined in Table XXI allows me only a very limited latitude I would believe that the leukemia patient had a prothrombin concentration of 85 percent of normal if the experiment was done in such a way as to compare it with the normal at the time the work was done and I would guess that the dicumarolized patient has a very low prothrombin but has sufficient Ac globulin present I would guess that

the barium sulfate plasma and the calcium phosphate plasma took out prothrombin and left the Ac globulin in solution That is about as far as I would care to go with that experiment Then I would keep in mind that it is very likely that we shall be able to find some thing to explain the data and do some good experiments to prove that something new must be a part of the explanation, of course that might take many years

Barker I believe that Dr Alexander has made some good points in commenting on the attitude of the clinicians toward this whole subject Primarily we are interested in the prevention of thrombosis by administering those drugs that impair coagulation but at the same time we do not want to impair coagulation sufficiently to cause bleeding In attempting to standardize administration of the drugs we are faced immediately with a great and often unpre dictable variability of response among different patients to the drugs The variability in response among different patients to fixed doses of dicumarol given orally as measured by the one stage prothrombin time is well known The variability is not only found among different patients but may occur in the same patient at different times Also it seems that a certain degree of prothrombin deficiency as indicated by a certain prothrombin time may not always indicate that thrombosis may or may not occur or that bleed ing may or may not occur in different patients or even in the same patient at different times This is particularly true when the first week of dicumarol therapy is compared with later periods of dicumarol therapy

In a general way we deal with two classes of patients when we use dicumarol We treat patients who have no potentially bleeding lesions and patients who have potentially bleeding lesions such as recent surgical wounds or gastrointestinal ulcerations When the usual standards for dosage are used based on the production of a certain effect as measured by the one stage technique the risk of bleeding is quite different in these two groups

The accumulated evidence much of which has been discussed at this Conference indicates that the anticoagulant effect of dicu marol is greater than its effect on prothrombin alone if we define prothrombin as the substance which is measured by the two-stage test There is some reason to believe that the effect is greater than is indicated by the one stage test which measures more factors than prothrombin alone The one stage test has been a valuable and practical guide to dicumarol therapy However in spite of some commendable results in large series of patients I do not think that

we can be too certain that our so called optimal levels of prothrombin deficiency as measured by the one-stage technique are always optimal I think the subject of optimal levels should be studied as much as possible by other techniques Some patients fortunately only a few are encountered who develop thrombosis and some patients also fortunately only a few develop bleeding even when the therapeutic prothrombin deficiency is optimal as measured by the one stage test Why? Other patients may have severe prothrombin deficiency as measured by the one stage technique and yet manifest no bleeding Why? I do not think the two stage test is the answer since it only measures one factor — prothrombin — and not the accelerators The answer may lie in fundamental differences among patients and disease processes which cannot be measured by any test or tests of the coagulating mechanism However there may be some cases at least where the explanation may lie in the degree of deficiency of certain accelerators which can be measured

Smith It seems to me the general trend of our discussion is in the direction of recognizing the multiplicity of variables involved in blood clotting This is a great step forward I am afraid there has been far too much dogmatism on the part of all of us with regard to substance subject matter and assay procedures A few years ago we thought we were dealing exclusively with thromboplastin calcium prothrombin fibrinogen fibrin but now we are confronted with the problem of the accelerator factor This is another critical variable apparently totally essential under normal physiologic conditions for the formation of a clot. There is a strong suspicion that there may be other variables Dr Mann has evidence he interprets as indicating a cofactor for thromboplastin Dr Quick comes forward with a suggestion not a new one but a repetition of what Bordet said many years ago that prothrombin exists in a precursor form In some species it is in one form and in another species it is mainly in a converted stage as fully activated prothrombin I think some of you have seen the note by Dr Owren (Owren P A A new previously unknown clotting factor *Scand J Clin and Lab Investigation* 1 162 (1949)) in which he claims but does not include data that prothrombin can be fully recovered from serum several hours old

In the light of all this I wonder how we can say that we have a method dealing exclusively with prothrombin I am willing for one to disarm on this whole problem What we need is more scholarship in the field and less dogmatism This is long overdue

the barium sulfate plasma and the calcium phosphate plasma took out prothrombin and left the Ac globulin in solution That is about as far as I would care to go with that experiment Then I would keep in mind that it is very likely that we shall be able to find some thing to explain the data and do some good experiments to prove that something new must be a part of the explanation of course that might take many years

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the term prothrombin test in the transactions of this meeting

Smith Use quotation marks

Wright How about parentheses?

Quick Probably the originator of the test ought to be consulted too

Wright You are here

Quick I make a predication irrespective of what action we take that it will remain the "prothrombin time" till the end of time or till at least we have passed out of existence but I would like to ask a practical question if I may? I think most of the men who have worked with the one stage method agree that a prothrombin activity let us call it that or whatever you want to call it of 20 percent of normal is the ideal level to approach Do you agree with that, Dr Barker?

Wright Therapeutically?

Barker On the basis of what test?

Quick On the one stage test!

Barker It has been so defined in publications if the 20 percent represents a comparable one stage prothrombin time to that noted on the dilution curve for 20 percent normal plasma

Quick In that case I would like a rather definite answer as to what level should be approached by the two stage method for therapeutic effectiveness I have been trying to find that data and I have not been able to do so

Alexander Does anybody know the answer to that? From the data in Table XXI it seems to me that we are not at all sure that prothrombin is the thing to be interfered with Here is a dicumarolized patient who still has 46 percent of his prothrombin by the two-stage method and yet has a prothrombin time of 40 seconds From clinical experience it would appear on the basis of this case that 46 percent of prothrombin by the two stage method is a satisfactory stage of interference with the clotting mechanism At least that is how I would interpret the data since a 40 second prothrombin time under the influence of dicumarol is generally considered a desirable range I think this raises a question at least in my mind as to whether reduction in prothrombin or whatever it is that is converted to thrombin that can clot fibrinogen is the significant thing I wonder whether the physiologic mechanism invoked for the prevention or treatment of phlebothrombosis may not be different from mere reduction in prothrombin Perhaps prothrombinopenia is merely an incidental phenomenon that we observe under the influence of these drugs

Neither do I think it is altogether helpful to speak of a promthrombin complex This problem gentlemen needs to be analyzed, not masked under pretty verbalizations The distinction and separation of the different variables should be made by chemical means and they should be studied for their separate effects The more analytical we are and the less dogmatic we are the further our knowledge advances

In the meantime I think our clinical advocates can avail themselves of any simplified test that is useful Why should they not be guided by pure expediency, when necessary? They are trying as far as possible to make their studies more and more specific and I think they are accomplishing this purpose

Wright I would like to concur in that opinion, Dr Smith I have believed for a long time that one of the major inhibiting factors in the progression of our thinking and particularly the thinking of those who have not examined the problem as carefully as the members of this group has been the nomenclature by which we have termed certain tests as "prothrombin tests" or "prothrombin time tests This has set up a wall against which we have been bumping our heads We have not been willing thus far to change to a name that is perhaps less specific but more accurate I would like to invite you to discuss this point I am not certain of the term of choice but we need another term that will call attention to the problem

Edsall Why should we not call the one stage test simply the one stage test or to make it more specific call it the one stage test with added thromboplastin?

Wright We would have to say one stage test for something because otherwise it might be confused with tests in other fields

Edsall Yes It is essentially a clotting time measured under certain defined conditions and specifically with the addition of one reagent thromboplastin

Brinkhous The term accelerated clotting time was used several years ago for the one stage test

Smith I doubt whether we are going to get away from the phrase prothrombin test The main thing is that we understand its limitations and that we agree that the test measures the summation of many variables that interact either simultaneously or in sequence I am quite prepared to say that I think the two stage test is subject to the same criticism

Wright If enough people thought that some other term might be more accurate we might start in a modest way by qualifying

the term prothrombin test in the transactions of this meeting

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Quick Probably the originator of the test ought to be consulted too

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Wright Dr Brambel do you consider that 46 percent represents what a two stage therapeutic level should be?

Brambel I am not prepared to comment on that phase In Table XXI we ran the two stage procedure for comparison and for the amount of data that we accumulated definite conclusions cannot be drawn

Wright What did you consider to be the therapeutic level?

Brambel I am not prepared to say at what level the percentage should be for a therapeutic level with the two stage method Our experience has been too limited

Wright Would somebody who uses the two-stage method comment? Dr Olwin you made comparisons and reported them here

Olwin To report our experience in the use of the different methods in the control of anticoagulant therapy Figure 34 shows Dr Quick s method and I believe we meet all of his requirements However since we have recently been studying Ac globulin and inasmuch as that factor is stable in citrated plasma and not in oxalated plasma we have shifted our whole system to citrated plasma In our experience the prothrombin time is slightly less with citrated plasma than with oxalated plasma With oxalated plasma the normal prothrombin time is between 11 and 13 seconds If our thromboplastin, which is acetone extracted rabbit brain does not meet those requirements it is not used With the citrated plasma the prothrombin time is between 10 and 11 seconds for normal plasmas These lines represent curves made by diluting samples of so called normal plasma with saline I am sorry that I do not have a figure to show the results of our tests with plasma diluted with deprothrombinized plasma because they do not in any way act as uniformly as do these curves Using the same plasma samples done the same day using the same deprothrombinized plasma for dilution we have curves that vary materially from these We used acetone dried rabbit brain here The modified two stage method was done on the plasma used in Figure 34 There values in percentage are given at the side of the figure

Alexander What is the normal unitage considered to be?

Olwin That may vary with the lot of reagents that you use We do not make an attempt to get 300 as a control prothrombin unitage each time We feel that if it is uniform over a period of time and we control it accurately, that any unitage between 275 and 400 is satisfactory because of this variability we choose to report the results in percent of normal We feel that the time and reagents wasted in throwing out lots that do not give a normal of

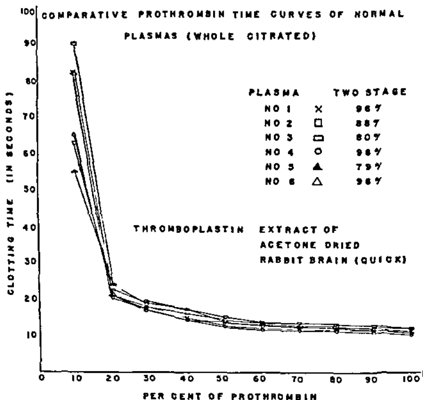


FIGURE 34

300 units is not worthwhile Inasmuch as we have tested this principle over a period of time we believe it is sound

Figure 35 simply enlarges the distance between 10 seconds and 30 seconds so that the figures in this bracket may be a little better examined You will note here that the figures do not quite meet the normal requirements This is citrated plasma The normal whole plasma prothrombin times are from about 10 5 seconds to 12 seconds One point I should like to bring out here is that the curve according to Dr Quick s original method is remarkably uniform with the different plasmas There are certain other points however that I think we should observe Plasma #3 has an 80 percent prothrombin by the two stage method and we find that at most dilutions it gives longer times than any of the other plasmas Plasma #5 however which has practically the same two stage prothrombin i e 79 per cent varies somewhat throughout the one stage curve Even though

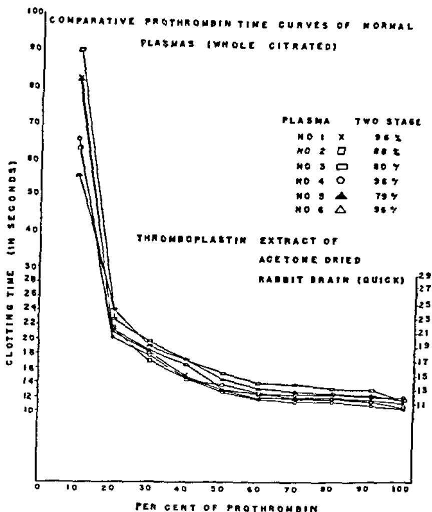


FIGURE 35

these variations are slight I think we must be aware of such variations in constructing control curves Even though the original whole plasma prothrombin times are relatively uniform for different plasmas the dilution curves may vary And in the bracket of 100 per cent to 20 percent of whole plasma a few seconds make a marked difference

Another point which I think we should emphasize here is that when the dilution goes to below 20 percent all the plasmas show a marked change in the prothrombin time Curves that have been relatively parallel in lower dilutions may diverge

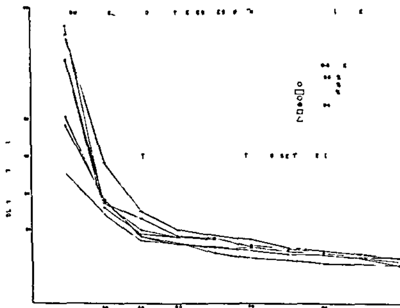


FIGURE 36

Recently there has appeared on the market made by Schieffin — and I express my appreciation to Dr Seegers for calling this to my attention — a thromboplastin which we have used both in the one-stage and two-stage tests with some satisfaction Heretofore we have at times tried the commercial preparations and have not found them satisfactory They vary appreciably and we have rejected them in favor of Dr Quick s thromboplastin which is the most uniform thromboplastin preparation that I know Here again as you see in Figure 36 the time for normal plasma is about 11 seconds It varies very slightly but in most plasmas that we consider within a normal bracket the time is reasonably uniform and we get almost the same type of curve that we obtained with Dr Quick s plasma Again there is the marked change as the 20 percent dilution is approached These are all whole plasmas and here we have included the two stage test figures which varies from 80 per cent to 96 percent of normal

Alexander You say the two stages in Figure 36 were done on whole undiluted plasmas?

Olwin Whole plasmas undiluted These are the original plasmas from which the curves were constructed It was whole plasma Of

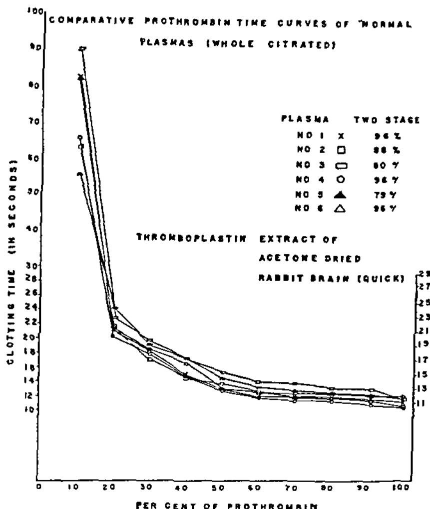


FIGURE 35

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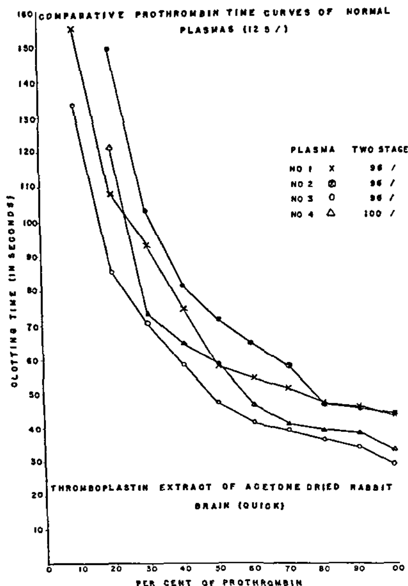


FIGURE 37

Olwin Figures 34 and 35 showed the same curves the second simply showing an enlargement in the bracket between 10 percent and 30 percent Figure 36 shows similar curves using extract of horse tissue instead of rabbit brain The points on the extreme right

course these plasmas were diluted with normal saline to get the various percentages and corresponding prothrombin times for the one stage test

Alexander I am in a bit of a fog The curve was constructed from whole plasma How do you get a 12 5 percent value for prothrombin in Figure 36

Olwin I think Figure 37 explains that Figure 37 was constructed with the original plasma being 12 5 percent of normal according to Dr Link s technique

Alexander With saline?

Olwin With saline and then another curve was constructed dilut ing this further in order to establish a prothrombin time curve Does that answer your question?

Alexander No

Tocantins I should like to raise the point as to whether or not the figures representing the plasma dilutions refer to dilutions of the original plasma A distinction should be made between the expression of dilution in terms of the original plasma and that of the actual clotting mixture Confusion on this point has led to a great deal of misunderstanding In the two stage method dilution of the plasma is always estimated on the basis of its actual concentration in the final clotting mixture On the other hand the one stage method is always spoken of as being done on 100 percent plasma while actually the plasma concentration in the final clotting mixture is about 27 percent It is an accelerated clotting time done on diluted plasma from the very start and not on 100 percent plasma The method using a so called 12 5 percent plasma is actually done on a plasma concentration in the final clotting mixture between 3 percent and 4 percent *

Flynn You mean the 12 5 percent does not include the three fold dilution that occurs in final clotting tube

Tocantins It does not The plasma concentration that is im portant to consider is that in the final clotting mixture and not in the original plasma

Olwin Here we are dealing in terminology Dr Tocantins has made a very important point

Alexander That bothers me but I am sorry for my stupidity I would like to clarify my own thinking if we can refer to the preced ing Figure 36

* Editor s note Dr Tocantins point is most pertinent It cannot be too strongly stressed

Flynn The ratio 11/10 is the same as 63/58 but in charting the latter a spread of ± 5 seconds seems much greater than a spread of ± 1 second

Olwin I see what you mean The ratios have not been calculated The point I wish to make is that using the same plasma using exactly the same technique using the same reagents preserved it is true but establishing the same criterion for the accuracy and standards for reagents we get a different curve on the same individuals

Quick How do you manage the fibrinogen? Does not that get pretty low?

Olwin Yes We have supplemented the fibrinogen in some of these and we find that the end point which is much clearer with fibrinogen added still is at the same time Without fibrinogen the end point is not a true clot though it is readily recognized

Flynn Within the limits of your experiment I doubt if the addition of more fibrinogen will appreciably change the clotting time but as you said it will change the character of the clot

Olwin We believe it is important to be critical of any test we use as well as of any new test Undoubtedly our tests are going to be improved from time to time and we would like to try each one as it comes along to see if it has advantages over those used previously When such methods are found then certainly we should discard our old ones in favor of the new ones When Dr Owren was here last year I talked with him to some extent about his one stage test and of the method of preparation of his reagents We set up his method in our laboratory in order to compare it with the tests that we were using His thromboplastin is fresh human brain extract The brain is removed from a fresh cadaver and is then triturated with slightly warmed saline about 40 I believe it is It is allowed to extract for two days then filtered and the extract frozen at −35 According to Dr Owren it will stay uniformly active for about three months In order to render certain other factors constant he uses as a menstruum beef plasma from which prothrombin has been removed To this the unknown plasma is added I think probably most of you are familiar with that technique The curves (Figure 38) Dr Owren makes use of are plotted entirely in the 0-10 percent bracket rather than 0 to 20 percent He dilutes his plasma originally to 10 percent and then establishes curves by diluting that in turn to 90 80 70 percent et cetera Figure 38 represents a group of curves established by that technique using plasmas with prothrombin levels according to the two stage method ranging

show the prothrombin times for different so called "normal" plasmas

Alexander What happened subsequently?

Olwin We have diluted those to 90 80 70 60 etc, percent of normal with normal salt solution On those various dilutions we have run duplicate titrations using the one stage technique as described by Dr Quick, but using an extract of horse tissue which is a commercial preparation

Alexander So that the 10 percent points represent one part of that whole plasma diluted with nine parts of physiological saline and then the prothrombin time is determined on that mixture?

Olwin That is right and all of these represent the same thing Now in Figure 37 we have diluted the plasma to 12 5 percent of normal according to Dr Link s technique and then we have again diluted to the concentrations shown with normal salt solution and have arrived at these curves

Alexander You consider then 100 percent down at the bottom one part of normal plasma with eight parts of physiologic saline, that is your 100 percent figure there?

Olwin Yes that is right

Again you can see the different plasmas give different curves a little more widely separated here but remember that the original dilution to 12 5 percent of normal puts the curve into the 0 to 20 percent bracket It is about at this point that you would normally expect the exaggeration of the original curve I might mention that Owren (Owren P A The coagulation of blood *Acta med Scandinav* 194, *1 (1947)*) makes a point of that in his one stage technique feeling that a wider spacing of prothrombin times provides for a greater accuracy in the results obtained Dr Link I believe is of the same opinion — hence his 12 5 percent plasma technique It is interesting that plasmas #1 and #2 are from the same individual These were done on different days using the same acetone dried rabbit brain solution but here there is a wide variation and I think it is interesting that such can occur in the same plasmas How to interpret that I am not sure but it seems to me that again we must have within individuals a wide variation of a number of different factors

Flynn In Figure 37 are the spreads with diluted plasmas proportional to the spreads that occur with more concentrated plasmas If a plasma clots at 10 seconds ± 1 second the same plasma diluted may clot at 58 seconds ± 5 seconds in which case the spread is proportional Have you calculated these ratios?

Olwin I do not understand the question

Olwin We simply ran a series of plasma with and without fibrinogen to see if the end point we were getting changed when more fibrinogen was added

Alexander Yes but how can you or how do you tell whether a particular blood specimen may or may not require the addition of fibrinogen?

Olwin The use of added fibrinogen is not a part of our routine one stage technique neither is the use of diluted plasma i e 12 5 percent or 10 percent We believe that such dilution does not in crease the accuracy of the test These are experiments that we ran to allow us to decide whether or not we should adopt these methods and for all of our one stage tests we use the orthodox method of Dr Quick with the slight variation that citrate rather than oxalate is added to the plasma at the time of collection

I think the clinician s need is for a test which will allow him to determine (a) what dosage of a drug is required (b) when the patient s prothrombin is therapeutically controlled and (c) when there is danger of bleeding In over 3 000 instances we have com pared the results of the one stage and two-stage methods using the same sample of blood on each occasion for the two tests Originally we used the unmodified two stage technique and in the past two years the modified technique i e with Ac globulin added The one stage technique is that of Dr Quick the thromboplastin being acetone dried rabbit brain In the early days we used oxalated plasma and now we use citrated plasma.

Undoubtedly we are affecting a number of factors when we give anticoagulants I don t think that there is any question about that at this time If we can get accurate control of the effect of the drug by measuring one of the factors that we are affecting it may be that that is the best criterion for controlling the administration of the drug Beyond that I would not want to make a statement but we must get some criterion for controlling the dose of the drug If we can affect one factor uniformly perhaps we should be guided by such effect At least that has been our feeling It is unimportant for the present discussion why we gave it but one patient (Figure 39) had dicumarol because of a thrombophlebitis He improved went home the signs and symptoms recurred and he came back That is the reason for the change in the curve during the early days but the point I should like to make here is that with a relatively uniform dosage of dicumarol this patient was maintained on a therapeutically effective dosage At least his thrombophlebitis re curred when his prothrombin rose and it did not recur when he was

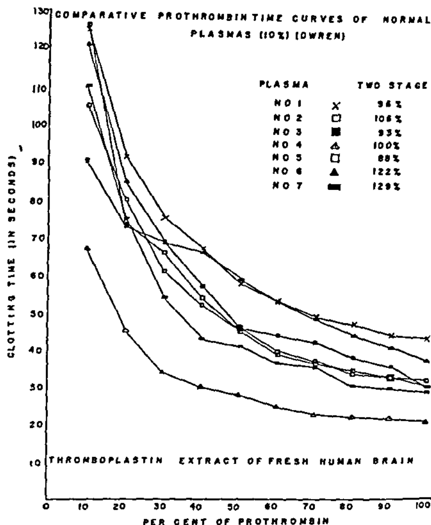


FIGURE 38

from 88 percent to 129 percent of normal As you can see, the plasma having the most prothrombin (#7) does not have the shortest time Neither does the one having the least amount of prothrombin (#5) have the longest prothrombin time Plasma #6 which has the second highest prothrombin level (122 percent two stage) has the second longest prothrombin time These seven so-called normal plasmas then when diluted to 10 percent of normal will give relatively widely varying curves by the one stage technique, using extract of human brain

Alexander May I ask when do you decide to add fibrinogen?

a bleeding bracket Hence we have felt that the two-stage test is the safest and is the most accurate method for measuring the prothrombin level when dicumarol is being administered

I agree with Dr Smith that as time goes along we are going to make changes in our program I think that we should test every new method that comes along to see if it offers advantages which are greater than those of the tests which are being used currently But for the past four years we have adhered to the two stage test and Dr Quick s original one stage test as the two that are most valuable in the control of dicumarol therapy

Alexander Dr Olwin referring again to Figure 39 am I correct in inferring that you state when the prothrombin drops below 10 percent by the one stage method that that is the time you take steps to rectify it? Do you have an observation there on April 10th as nearly as I can interpret from the data where the one stage shows 100 percent?

Olwin April 10th The one stage shows 100 percent

Alexander Sixty percent by the two stage method?

Olwin Yes

Alexander Then you have observations further along where the two stage value is 50 percent of normal yet by the one stage method the value is approximately 20 percent

Olwin Yes about 15 percent at May 19th in Figure 39

Alexander Yes Similar observations along there where there is that wide discrepancy You always feel that the 10 percent value by the one stage is critical as a value even though the two stage may not be in accord with that?

Olwin I would say that it is very rare that the two stage test is higher than the one stage except as Dr Barker and his associates pointed out several years ago that the one stage drops more rapidly than the two stage in the early days of dicumarol therapy Perhaps during the first two days of therapy the one stage will be higher than the two stage and after he pointed that out we went through our records and found out that in practically all our cases that was true To answer your question more directly — except for the above mentioned period — the two stage estimate of prothrombin is nearly always lower than that of the one stage In that the one stage is a measure of factors other than prothrombin we feel it gives information as to the safety margin present when the prothrombin has been reduced to below 10 percent according to the two stage test As long as the one stage gives an estimate of above 10 percent bleed

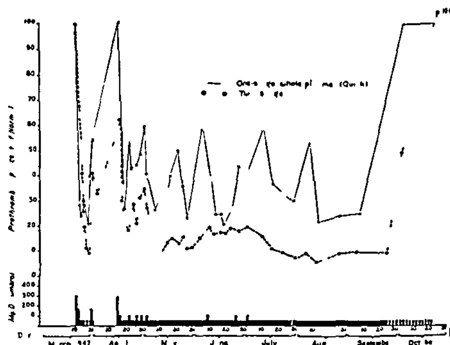


FIGURE 39

maintained on a level somewhere between about 8 percent and 20 percent of normal We believe (and at long last I am attempting to answer Dr Wright s question) that ideally the prothrombin as measured by the two stage test should be maintained between 10 percent and 20 percent of normal Almost routinely in our laboratory according to the two stage method it drops below 10 percent and when that occurs the one-stage is run on that plasma. If that is found to be above 10 percent we are not concerned about possible bleeding If however, it is found to be below 10 percent (one stage) we then institute measures to restore the prothrombin to a safe level

I believe that the one stage test is a summation of the clotting factors and as such is a most valuable test You can see in Figure 39 that the prothrombin according to the two stage test remained relatively uniform and we would assume when a relatively uniform dosage of the drug was being given that this would be a reasonable result to expect The one stage test on the other hand showed rather wide variations If on occasions when the one stage test showed high levels we had given dosages of dicumarol which we might have given had we accepted these figures for the prothrombin level we probably would have thrown the patient into

Quick In regard to thromboplastin and its estimation one can get clotting time values in the same way as in the prothrombin determination by the one stage method The prothrombin time is kept constant while the thromboplastin concentration is varied The curve one obtains can be expressed by the equation

$$ct = a + \frac{k}{C}$$

(c t = clotting time C is concentration of thromboplastin and a and k are constants) If the work is done carefully it is surprising how well the theoretical values agree with the experimental ones down to 10 percent Beyond that the values are not reliable The minimum amount of acetone dehydrated rabbit brain which will yield a prothrombin time of 12 seconds for human plasma is designated as 100 percent

Tocantins Would you say Dr Quick that that empirical formula describes the reaction of plasma to an excess of thromboplastin added to it?

Quick I don t know what it means but it is remarkable that there is good agreement between the calculated values and the experimental values

Tocantins If plasma in high concentration is used the experimental values do not agree with those calculated with that empirical formula Moreover the curve expressing the relation of the concentration of plasma to accelerated clotting times is not hyperbolic it is parabolic

Smith I would agree that the whole problem is empirical. The mere fact that it corresponds does not mean that it signifies any underlying mechanism as chemical reactions are ordinarily described by mathematical equations Dr Quick originally published that formula with only one constant Then he found it was necessary to put in two constants If one adds enough constants one can make almost any curve develop in a prescribed way Perhaps next year he will have a third constant in and then it will work with concentrated plasma

Tocantins In concentrated plasma at least three constants are needed

Smith The mere fact that it fits the experimental data means one found a formula which fits the data in the note book

Quick This equation incidentally also fits the reaction of thrombin and fibrinogen and it also fits if you make prothrombin calcium constant thromboplastin so that we do not need three constants

ing will rarely occur Hence, from the standpoint of safety, it is a critical level, regardless of the two stage estimate

Alexander Then the experiment of Dr Brambel s in Table XXI makes sense, since the value obtained with the two-stage method is higher than that obtained by the one stage method.

Olwin Very definitely

Mann I would like to say a few words regarding the Schieffelin thromboplastin which Dr Olwin used because I have had a little experience with this thromboplastin, and it has most unusual properties At the beginning of the work on anticoagulant therapy at the Mayo Clinic Miss Hurn, Dr Barker and Dr Magath had certain thromboplastins that gave the same times with normal plas mas yet behaved quite differently with certain abnormal plasmas particularly the plasmas of individuals treated with dicumarol Thus we may say that certain thromboplastins are insensitive to the changes produced by dicumarol the Schieffelin thromboplastin is a striking example of such insensitivity Patients in the initial phase of the dicumarol effect may have a prothrombin time of 70 seconds or so with the version of the Quick technique which we use but less than 30 seconds with the Schieffelin thromboplastin I am sure that this thromboplastin would be quite safe in Dr Olwin s hands, but I would hesitate very strongly to recommend it for use gen erally by persons with little experience in the performance of coagulation tests

Smith Do you know, Dr Mann how they make this new type of thromboplastin?

Mann I would like to know It is from horse tissue

Olwin Possibly it is a trade secret Perhaps Dr Seegers knows

Seegers I don t know One of my graduate students smelled the Schieffelin product and decided it contained phenol

Mann There is phenol in it

Seegers He decided that our own lung extracts which we have been making for 10 years or more could be stabilized by putting in some phenol So far the addition of phenol has kept them stable for about three months in the ordinary ice box

Mann I am also interested in the source of this thromboplastin because in our experience such striking insensitivity to the effect of dicumarol has been exhibited only by viper venom by placental thromboplastin and by brain preparations which have been treated with dilute serum Some of the Schieffelin preparations were strongly blood tinged and their behavior certainly differed from that of the usual brain thromboplastins

COMPLEXITY OF THE EFFECT OF DICUMAROL

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When Dr Barker and his colleagues began to use dicumarol clinically and wished of course laboratory control Miss Margaret Hurn worked out the technique which is employed at the Mayo Clinic it is essentially a slight modification of Dr Quick s original method In setting up this procedure Miss Hurn made this curious observation that two given thromboplastic extracts which gave identical prothrombin times with normal plasma might differ decidedly when tested with certain dicumarol plasmas[1] At that time there was no explanation of this effect which was quite definite although not manifest with all thromboplastins and all dicumarol plasmas It seemed that special controls were indicated to maintain reproducibility of prothrombin times on dicumarol plasma although it was not then known to what the reproducibility referred Dr Quick s original thromboplastin is somewhat more stable than the acetone dehydrated variety the extracts maintaining constant activity for several days in an ordinary refrigerator Hence we were able to compare each thromboplastic extract with a standard one previously used and in this way constantly maintain a stock of thromboplastin with constant properties with respect to dicumarol as well as to normal plasma

Then later when Miss Hurn and I[2] became interested in studying dicumarol plasma by the two-stage method it was soon apparent that early in the course of dicumarol therapy there was often considerably more prothrombin in the plasma than one would expect on the basis of the "percentage normal prothrombin calculated from dilution curves of normal plasma It was about that time that general interest was aroused in the concept that some factor other than prothrombin may affect the rate of conversion of prothrombin to thrombin One might at first think that if there is more prothrombin in dicumarol plasma than would be expected from the Quick prothrombin time this might be due to deficiency of a prothrombin conversion factor We did not draw that conclusion at

These two constants are sufficient and it can be calculated by the law of least squares

Flynn It certainly does not fit small amounts of thrombin

Quick It is in the range where the change of the curve is most marked

Brambel What was the concentration of the calcium chloride that you used with the Schieffelin?

Olwin Fortieth molar calcium chloride

Brambel Is that what the Schieffelin people recommended?

Olwin No

normal. Hence we believe that a considerable portion of the effect of dicumarol as measured by the one stage prothrombin time is not accounted for by changes in either prothrombin or labile factor but is due to some other factor or factors[5] Apparently the question must be asked as to whether these findings may not apply to these other related anticoagulants

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DISCUSSION

Flynn Dr Mann do you use the same thromboplastin in your two-stage method that you use in your one stage method?

Mann No for the two stage method we use acetone dehydrated thromboplastin For the one stage method we use Quick s original thromboplastin which is not acetone-dehydrated

Flynn Can you demonstrate the co thromboplastin effect with acetone-dehydrated thromboplastin?

Mann Yes

Flynn Do you add your co-thromboplastin factor in the form of serum to the two-stage?

Mann We have to add the serum in the two stage method when we are dealing with dicumarol plasma

Often with dicumarol plasma we are unable to obtain what we think is a quantitative yield until we add serum in the two stage method We check the serum as rigorously as we can to be sure it does not contain any significant quantity of prothrombin

Alexander Human or bovine?

Mann Human

the time because we were impressed by the fact that it was actually in dicumarol plasma that Dr Quick obtained the first direct demon stration of a prothrombin conversion factor, the factor which he first called "prothrombin A," which has apparently since had various different names such as labile factor, Ac globulin and Factor V No one has been able to demonstrate a decrease in this factor detectable by the one stage method following the administration of dicumarol Using Seeger s method for the determination of Ac globulin a relatively small decrease in this factor was found by Fahey et al[3] During the rapid production of a marked dicumarol effect in rela tively sensitive individuals there is commonly a striking change in the one stage prothrombin time while the two stage prothrombin level may remain nearly normal We do not believe that this large change in the one stage prothrombin time can be explained by a deficiency of Ac globulin which cannot be detected by one stage methods Instead we think the explanation involves some other deficiency in the plasma

When one does the two stage determination on normal plasma it has been observed that there is a preliminary lag in the conversion of prothrombin to thrombin With the technique we use for this test this effect is marked so that only traces of thrombin form in the first minute of treatment with thromboplastin while ten times as much thrombin may form in the second minute as in the first If the thromboplastin (which for this purpose is made from acetone dehydrated rabbit brain) is pre incubated for three minutes with very dilute plasma or serum (around 1 600 in the total volume of the system) and then the usual amount of plasma (1 100 dilution) is added much more conversion of prothrombin to thrombin occurs in the first minute This effect we have made the basis for a test which we call the co thromboplastin assay"[4] since what is actually measured is an increased thromboplastic activity When tested in this manner dicumarol plasma with a highly prolonged prothrombin time consistently shows marked deficiency of co thromboplastin even though considerable quantities of prothrombin may be present Aged plasma on the other hand which has a very prolonged pro-thrombin clotting time due to deficiency of labile factor shows normal co thromboplastin activity Co-thromboplastin deficiency may be demonstrated by the one stage method also If the throm boplastin extract used in the one stage test is briefly exposed to very dilute serum (1 300 in the total system) the prothrombin time of those specimens containing nearly normal prothrombin but little co thromboplastin is brought from very abnormal figures nearly to

Alexander I would not but apparently the terms are being used synonymously But as I gather if I interpret Dr Mann s remarks correctly he is adding human serum four or five hours old which according to Dr Seegers should be devoid of Ac globulin Am I correct Dr Seegers?

Seegers We have published something on that. In fact it is an original observation which we are quite happy about (Murphy R C Ware A G and Seegers W H Stability of serum Ac globulin *Proc Soc Exper Biol and Med* 69, 216 (1948) Murphy R C and Seegers W H Concentration of prothrombin and Ac-globulin in various species *Am J Physiol* 154, 134 (1948)) In human serum Ac globulin concentration decreases rapidly That is true for many other species but not for the cow and rabbit

Alexander I would quite concur with Dr Seegers From our studies it would seem therefore that the effect that Dr Mann has found is not attributable to Ac globulin as such In that connection I might cite some observations we have made (Landwehr G Lang H and Alexander B Congenital hypoprothrombinemia *Am J Med* VIII 255 (1950)) regarding congenital hypoprothrombinemia where the child presumably had no appreciable prothrombin by any technique and yet showed a plasma which was quite different from dicumarol plasma containing the same order of magnitude of prothrombin by the one stage technique So I am in complete accord with Dr Mann that dicumarol does affect some thing other than prothrombin in addition to prothrombin

Mann With regard to Dr Olwin s question I don t think that we can conclusively rule out the fact that Ac globulin may play a role in the effect. I would merely say this the conversion defect in the early stage of dicumarol therapy in our experience is a very great one indeed When one has a person with a prothrombin time of 100 seconds and 200 units of prothrombin demonstrable in the plasma as we have found on a number of occasions that is a great defect in conversion It seems to us that if the defect were on the basis of a deficiency in whatever that substance is which is lost when oxalated human plasma is stored it should not be possible to take aged oxalated human plasma with a prothrombin time of 100 seconds and mix it with that dicumarol plasma and get a practically normal prothrombin time Yet that is what Dr Quick did in 1943 and he has subsequently reported that he can demonstrate no change in labile factor or whatever we shall call it I am not implying that these things are proved to be identical but one has to call them something I think it conceivable that there might be a small

Flynn Do you add Factor V?

Mann We do not add it

Flynn Is human serum aged or aged plasma?

Mann It is human serum obtained by spontaneous coagulation and is ordinarily four to eight hours old

Flynn That contains Factor V

Mann Some yes

Flynn How do you know your factor is not Factor V?

Mann The principal reason is the absence to the best of our knowledge, of any marked deficiency in Factor V in the dicumarol plasma We *have not done all possible tests, but certainly it has* been found that the amount of Factor V in human serum of that age is relatively not very great and the deficiency of Factor V in the dicumarol plasma is equally not very great

Olwin How old is the dicumarol plasma?

Mann Ordinarily the test is done on the same day

Olwin How long have the patients been on dicumarol?

Mann We have studied them at various times This effect is most striking when the patient has been on dicumarol therapy for only a few days or else there is a period of increasing dicumarol effect.

Olwin You mean after say three weeks the effect is greater with serum than in the early period?

Mann I am afraid I don t mean that at all I mean in the initial or increasing phase of the effect of dicumarol there is a lot of prothrombin demonstrable During initiation of dicumarol therapy in relatively sensitive individuals when we can get a marked increase in the Quick prothrombin time with very little if any change in the two stage determination the yield of prothrombin may be increased sometimes by the addition of human serum

Olwin We have found that Ac globulin or Factor V diminishes in the early stages of dicumarolization and in most cases studied returns to normal by the end of three weeks therapy If I properly understand you I would think the effect of serum could be ex plained very easily on the basis that you are adding a certain ade quate amount of Ac globulin We have found on a number of patients th it the Ac globulin is fairly promptly lowered when dicu marol is given

Alexander Isn t it true that human serum is quite devoid of Ac globulin? According to Dr Seegers it is not demonstrable in human serum obtained 4 5 hours after blood has clotted

Flynn Are you using the terms Factor V and Ac globulin synonymously?

Mann I have done very little with dicumarol serum My impression is that a lot of prothrombin remains in it. Dr Quick has recently reported that the consumption of prothrombin as measured by his prothrombin consumption test is impaired after dicumarol therapy That to my way of thinking is in agreement with the observation that there is a conversion defect in dicumarol plasma.

change in Ac globulin To the best of my recollection in your publications you have reported changes of the order of magnitude of a 50 percent decrease or less To our way of thinking a 50 per cent decrease in virtually any blood coagulation factor should not be expected to alter greatly the Quick prothrombin time If this conversion defect is due to a deficiency similar to that of aged human plasma it should be demonstrable by direct mixing experiments similar to those which originally demonstrated the deficiency of aged plasma which is not the case in our experience Dr Quick did not find it and there have been other workers who did not find any change on direct mixing experiments Therefore we think that deficiency of the labile factor is not the explanation of the conversion defect in dicumarol plasma

Flynn Can you show the conversion defect in your two stage method? For example how long does it take one unit of prothrombin to convert?

Mann In the early experiments which Miss Hurn did and reported in 1947 she used the ordinary two stage determination without adding any serum In the early phase of dicumarol therapy there might be two or three times as much prothrombin present as would be expected on the basis of the percentage figure from the dilution curve The minimal clotting time in such plasma frequently was obtained perhaps after 10 or 15 minutes which is certainly not what would be expected with normal human plasma It is more likely to be about three or four minutes

Flynn You think the discrepancy on the two tests is explained by the difference in the conversion rate?

Mann I am not so sure that is the whole story It might be that alterations in fibrinogen or the thrombin destroying factors might enter into it The main point we wish to make is that both the one stage prothrombin time test and the effect of dicumarol appear to be complex phenomena

Smith Dr Mann as a control for some of your experiments perhaps you have used canine plasma or serum because as Dr Quick and others have shown it is high in the labile factor and it might be possible to differentiate between the labile factor and some other factor

Mann We have not done that We have occasionally used bovine serum and curiously enough it isn t as good as human serum in increasing the yield of dicumarol plasma

Alexander Have you tried dicumarol serum?

TABLE XXVII†

THE EFFECT ON THE PROTHROMBIN TIME WHEN NORMAL HUMAN PLASMA IS PROGRESSIVELY DILUTED WITH HUMAN AND WITH RABBIT PLASMA FROM WHICH COMPONENT A WAS REMOVED BY ADSORPTION WITH TRICALCIUM PHOSPHATE

Normal human plasma (c c)*	0 1	0 09	0 08	0 07	0 06	0 05	0 04	0 03	0 02	0 01	0 00
Normal human $Ca_3(PO_4)_2$ plasma (c c)	0 0	0 01	0 02	0 03	0 04	0 05	0 06	0 07	0 08	0 09	0 10
Prothrombin time (sec)	12	12	12	12	12	12	13	15	18	26	∞
Normal human plasma (c c)*	0 1	0 09	0 08	0 07	0 06	0 05	0 04	0 03	0 02	0 01	0 00
Rabbit $Ca_3(PO_4)_2$ plasma (c c)	0 0	0 01	0 02	0 03	0 04	0 05	0 06	0 07	0 08	0 09	0 10
Prothrombin time (sec)	12	$11\frac{1}{4}$	$11\frac{1}{3}$	$11\frac{1}{2}$	$11\frac{3}{4}$	12	13	15	18	40	∞

† Quick A J and Stefanini M The concentration of component A in blood its assay and relation to the labile factor *J Lab and Clin Med* 34 973 (1949)

* The blood was collected in a silicone-coated syringe and transferred to a silicone-coated test tube containing sodium oxalate

THE INTERRELATIONSHIP OF PROTHROMBIN, PROTHROMBINOGEN AND LABILE FACTOR

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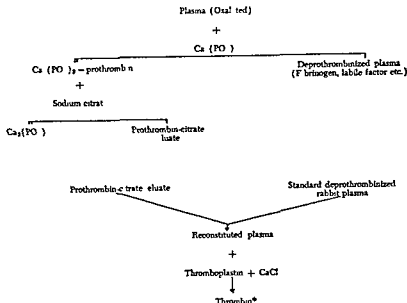
There are only two basic methods for determining prothrombin at the present time * the two stage and the one stage procedures All the modifications that have been introduced have added little of significance

For the sake of clarity it is desirable to define the essential terms I shall read to you my definition of prothrombin It is that con stituent of the plasma which reacts with thromboplastin calcium and labile factor stoichiometrically to form thrombin It is diminished in vitamin K deficiency and after the administration of dicumarol It is low in the two types of congenital hypoprothrombinemia which I have described[1] It is absorbed by tricalcium phosphate"

The second term is labile factor I think I am justified in naming and using the term labile factor because I discovered and de scribed this factor in 1943 At that time I also predicted that a clinical condition would probably be found in which this constituent might be deficient The following year Owren described such a case He found a patient in whom this factor was so low that it produced a hemorrhagic tendency Let us define the term labile factor It is a constituent of the plasma essential for the formation of thrombin which disappears on storage probably due to oxidation Its instability is accentuated by depression of the calcium ions Only a trace of it is adsorbed by tricalcium phosphate and some what more by aluminum hydroxide It is not diminished in vitamin K deficiency nor by dicumarol

It is of basic importance to know whether the labile factor is an accelerator or not *To this end I am presenting Table XXVII in* which the effect of adding deprothrombinized human and rabbit plasma to normal oxalated human plasma was studied It will be noted that the addition of deprothrombinized rabbit and human

* Editor s note Dr Quick overlooked the original contribution by P Fantl and M H Nance Relationship between Plasma Coagulation time and Prothrombin Concentra tion *Australian J Exper Biol and Sc 25 95 (1947)*



Measured by the prothrombin time

FIGURE 40

The determination of prothrombin by adsorption with tricalcium phosphate and elution with sodium citrate

deficiency after dicumarol administration in congenital hypoprothrombinemia after liver injury and for the amount remaining in serum i e in the prothrombin consumption test. The results have been so satisfactory and the correlation with the one stage method has been so excellent that we have a renewed faith and confidence in the one stage test. Equipped with two independent methods for estimating prothrombin it is now possible to consider the state and concentration of prothrombin in human plasma

A number of years ago I obtained results that I reported at one of the Federation meetings which I want to show you Table XXVIII It will be observed that when oxalated human plasma is stored at 5 C i e in an ordinary refrigerator a progressive increase in the prothrombin time occurs which means that the prothrombin activity decreases When sodium citrate is used and one part of 0 2 M added to 9 volumes of blood a similar drop in prothrombin activity is obtained When 0 13 M sodium citrate is used which is still adequate to make the blood totally incoagulable a strikingly different result is obtained. Instead of the prothrombin time becom

plasmas gave identical results Yet, rabbit plasma, according to the findings of both Dr Seegers and his group and of Dr Stefanini and me, contains much more of this new factor — call it labile factor, Ac globulin or Factor V If this agent were an accelerator deprothrombinized rabbit plasma which contains 50 times more than human plasma should show a much greater effect than human plasma which it clearly does not do This is not in accordance with the action of an accelerator In carrying out this experiment blood was collected in a silicone coated syringe with a needle similarly treated and kept in a silicone coated test tube immersed in an ice bath The prothrombin was removed by adsorption with tricalcium phosphate In this type of study great care is necessary in order to get accurate values Thus under absolutely perfect conditions a prothrombin time of 12 seconds instead of 11½ seconds should be obtained with deprothrombinized rabbit plasma

Smith In Table XXVII the prothrombin is decreasing as you pass from left to right in that chart?

Quick Yes but that does not change the essential findings namely that the prothrombin time remains uninfluenced by a large excess of labile factor Rabbit plasma with a concentration of 50 times more labile factor than human plasma does not shorten the prothrombin time below the fixed minimum of 11½ seconds This then is strong proof that the labile factor is not an accelerator

It became obvious that to clinch the matter we had to approach the prothrombin question from a different angle It seems strange that after all the modifications that have been added to both the two and one stage procedure no one went ahead and introduced a method new and different in principle Dr Stefanini and I had to do the job We observed that when oxalated plasma is treated with tricalcium phosphate the prothrombin is quantitatively adsorbed and furthermore that sodium citrate elutes it completely from the adsorbent When the eluate is added to deprothrombinized rabbit plasma the reconstituted plasma has a prothrombin time which is a direct measure of the prothrombin concentration of the eluate This new procedure for determining prothrombin which is presented in Figure 40 meets the objections which were often raised in regard to the one stage test Deprothrombinized rabbit plasma is an ideal medium for assaying the eluate since it has a high concentration of labile factor and it is uniform in composition The quantity of any hypothetical prothrombin conversion factors should be constant I might say in passing that we have used this method for determining the prothrombin in vitamin K

ing prolonged it actually becomes shorter This result puzzled me greatly and it was not until about a year ago that I found what I believe is the answer I shall try to develop the series of studies which lead to the answer I want to take this opportunity to give credit to Dr Stefanini who assisted me in this investigation

A valuable clue was obtained from the behavior of hemophilic blood When the prothrombin consumption time is done on hemophilic plasma or blood i e the prothrombin time of the serum it is found that it ranges from 7½ to 12 seconds It is remarkably constant for anyone hemophilic and the value does not change in 24 hours That it is a true value for prothrombin is vouched for by the fact that when the prothrombin is determined by the adsorption and elution technique the results agree with the one stage prothrombin consumption test When a prothrombin of 8 or 9 seconds is obtained on the serum it means that the prothrombin activity is greater than in the original plasma In thrombocytopenia a similar finding is made as shown in Table XXIX There is however a slight increase in the serum prothrombin time as the serum stands These results were very puzzling and no doubt some hoped that they would give the coup de grace to the one-stage method Actually these results have opened new avenues and now a satisfactory explanation can be offered For this we need Figure 41

You will remember that I showed you my scheme of coagulation last year It has since become slightly more complicated According

TABLE XXIX

PROTHROMBIN CONSUMPTION TEST

Subject	*Tube*	Minutes after the formation of a solid clot: 15	30	45	60
		Prothrombin consumption time in seconds			
Normal	1	8*	26	25	26
	2		8½*	26½	26½
	3				13*
Hemophilic	1	7*	8	8	8
	2		8*	8½	8
	3				8*
Thrombocytopenic	1	8*	9	10	11
	2		9*	10½	12
	3				8*

Determination made immediately after centrifugation

TABLE XXVIII

EFFECT OF DECALCIFICATION OF PLASMA ON THE PROTHROMBIN TIME

Agent of decalcification*	PROTHROMBIN TIME IN SECONDS								
	DURATION OF STORAGE								
	0 hr			24 hrs			48 hrs		
	I	II	III	I	II	III	I	II	III
Sodium oxalate 0 1 M	12	11½	12	15½	13	15	18	19	20
Sodium citrate 0 2 M	12	12	11½	14½	15	12	22	19	16
Sodium citrate 0 13M	12	11	11½	10½	10	9½	12	12	11

* One volume of the agent was mixed with nine volumes of blood

that about one fourth of the total prothrombin is free or reactive and that the remainder is in the precursor state

Tocantins Are these figures you just gave based on weights of the two relative substances?

Quick No the findings are based on prothrombin times What is necessary for the conversion of prothrombinogen to prothrombin? The only factor so far known to accomplish this is a rough surface such as glass If oxalated plasma or for that matter citrated plasma carefully collected to prevent any incipient clotting is put in a silicone coated tube and then stored in a refrigerator the prothrombin time will decrease steadily but if to this plasma labile factor is added in the form of deprothrombinized rabbit plasma the prothrombin time is restored to 12 seconds If on the other hand the oxalated blood is put in a glass tube and stored and then labile factor added the prothrombin time will be 8 to 9 seconds The most satisfactory explanation for these results is that in silicone the prothrombinogen is not changed to active prothrombin and therefore when labile factor is added the normal prothrombin time of 12 seconds is restored In the case of plasma stored in glass the prothrombinogen is all activated to prothrombin and therefore when labile factor is added the prothrombin time is very short because the free or active prothrombin has been markedly increased It is probable that prothrombinogen is prothrombin combined with an inhibitor which in contact with glass is removed When fresh oxalated human plasma is treated with tricalcium phosphate and the prothrombin eluted from the latter reagent with sodium citrate according to the standard technique of Quick and Stefanini[3] the eluate will have a prothrombin time value of 12 seconds When the eluate is stored in a glass test tube its prothrombin time value decreases to 8 seconds This shows that tricalcium phosphate adsorbs both prothrombin and prothrombinogen The eluate stored in a silicone coated tube retains the prothrombin time of 12 seconds

If hemophilic blood or plasma is allowed to coagulate in glass the prothrombin time of the serum is usually 8 to 9 seconds but if the plasma is put in a silicone coated tube the serum which is eventually obtained will have a prothrombin time of 12 seconds Why the difference? In hemophilia the thromboplastinogen is lacking therefore little prothrombin is consumed during coagulation In glass all the prothrombinogen is converted to active prothrombin therefore the serum will contain much more free prothrombin than did the plasma This accounts for the marked decrease in the prothrombin time In the silicone tube no prothrombinogen becomes

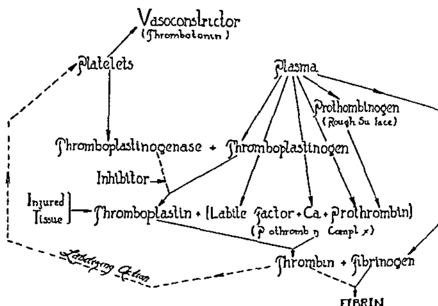


FIGURE 41
The Coagulation Mechanism

to the outline the plasma still furnishes thromboplastinogen the factor lacking in hemophilia labile factor, calcium in a combined form (but to what compound it is combined is still an engima) and prothrombin The formation of thrombin requires the interaction of thromboplastin labile factor, calcium and prothrombin stoichiometrically When any one of the first three factors is lacking the conversion of prothrombin is incomplete I have never obtained any results which would make it necessary to postulate a prothrombin conversion or accelerator factor It is logical to ask at this time why did Dr Mann get his peculiar results which prompts him to postulate an additional factor, and why did Dr Smith and I argue about the one and two stage methods? I think we now have the answer which is that in human plasma the prothrombin exists in two forms It is present in an active state which is the classical prothrombin the concentration of which determines the prothrombin time In addition there is inactive prothrombin which I have named prothrombinogen It can under certain conditions be converted to the active form This takes us back to the work of J Bordet and L DeLange[2] who postulated that all the prothrombin in circulating blood was in an inactive state which he designated as proserozyme We differ only in that our data indicate

How prothrombinogen is converted in the body to free prothrombin remains an unsolved problem

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converted to free prothrombin therefore the prothrombin of the hemophilic plasma and serum remain essentially the same, and the prothrombin time of both is 12 seconds

In thrombocytopenia or when the platelets are removed by high centrifugation the plasma on clotting will behave very much like hemophilic plasma because again thromboplastin is lacking and little prothrombin will be consumed in the clotting reaction Of course the cause of the lack of thromboplastin is the inadequate supply of the activator of thromboplastinogen If platelet poor plasma clots in glass the prothrombin time of the resulting serum will be 8 to 9 seconds This offers a means to determine total pro thrombin For this test blood is collected with the silicone tech nique and centrifuged at high speed to remove the platelets The platelet poor plasma is allowed to coagulate in a glass test tube In this clotting reaction very little prothrombin is consumed but all of the prothrombinogen is converted to free prothrombin The prothrombin of the serum becomes a measure of the total prothrombin For normal human blood a serum prothrombin time of 7½ to 8 seconds is usually obtained You will note that this value is not very different from the prothrombin time of normal dog plasma which is 6 seconds You will recall that whereas the one stage method shows a great difference between the prothrombin concentration of dog and human plasma the two stage method does not This can now be easily explained With the one stage procedure only free or active prothrombin is determined while in the two stage method most of the prothrombinogen is converted to free pro thrombin during the various manipulations Therefore the latter method measures essentially total prothrombin Since rabbit and dog blood contain little prothrombinogen the one and two stage methods agree well when compared on these bloods It is with human blood that difficulties arise because much of the prothrombin is in the precursor stage

Naturally the question arises what is the physiological purpose and significance of prothrombinogen Frankly I do not know Cer tainly there is no rough surface such as glass in the body which will permit the inactive prothrombin to be converted to the free state so it is questionable whether the inactive prothrombin par ticipates directly in the coagulation mechanism Can prothrom binogen function as a reserve? We have found that after the ad ministration of dicumarol the free prothrombin drops first and only after several days does the prothrombinogen begin to go down

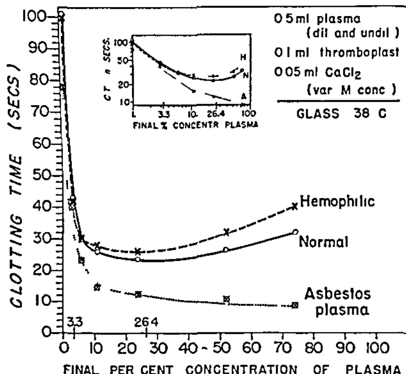


FIGURE 42

Dilution curves of three types of plasma activated by a strong thromboplastin. Inset The same curves plotted on logarithm scale Asbestos plasma obtained by allowing hemophilic plasma to stand in contact with asbestosis fibers without shaking for one hour at 20 C (10 mg asbestos fibers 1 ml. plasma) The plasmas were handled by the silicone technique until just before placing them in glass tubes. The concentration of prothrombin and Ac globulin of the three plasmas tested by the two-stage method (1) (2) were within normal limits Plasmas obtained from citrated blood (0 2 ml. 19% citrate to ml. of blood hematocrit 38) Dilution of the plasma in the first clotting mixture $\frac{60}{62} \times \frac{50}{65}$ or a starting plasma concentration of 74 percent. Calcium concentrations for optimal clotting used throughout

r p m for 1 hour at 5 C) from blood collected with special pre cautions by the silicone technique Plasmas were not allowed to come into contact with glass until the actual testing Unless these precautions are taken the results are erratic The greatest dif ference between the three types of plasma is evident in the clotting mixtures of high plasma concentration As the hemophilic and normal plasmas are diluted their clotting times shorten Very little change in the clotting time of each plasma is observable at con

INFLUENCE OF THE ACTUAL PLASMA CONCENTRATION IN THE FINAL CLOTTING MIXTURE ON THE RESPONSE OF NORMAL AND HEMOPHILIC PLASMAS TO THROMBOPLASTIN IMPLICATIONS IN REGARD TO ONE STAGE 'PROTHROMBIN TIME' TESTS*

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What we have to present today deals with the importance of considering the actual plasma concentration in the final clotting mixture as a factor in determining the response of that plasma to clot accelerating agents such as thromboplastin cephalin platelets etc We shall attempt to show that

a) The familiar activity curves for various dilutions of normal plasma activated with thromboplastin according to the one stage method refer to the original plasma concentration and not to the final concentration of the plasma in the clotting mixture All one-stage prothrombin time methods are done on diluted plasma

b) The curve expressing the response of various dilutions of plasma to a strong thromboplastin follows a parabolic and not a hyperbolic course High plasma concentrations *slow* the response of the plasma to thromboplastin

c) The more stable the plasma is (e g hemophilic plasma) the more striking is the parabola The less stable (e g hypercoagulable asbestos plasma) the more the curve will approach a hyperbolic course

d) Significant differences in response to thromboplastin between hemophilic and normal plasma can be demonstrated if tests are carried out at a high plasma concentration

If a strong solution of aqueous extract of human brain is added to normal hemophilic and asbestos plasma beginning with a concentration of the plasma of 74 percent down to 1 percent curves such as those illustrated in Figure 42 are the result Unless otherwise stated all plasmas were separated by centrifugation (3 000

* Aided by a Grant from the U S Public Health Service

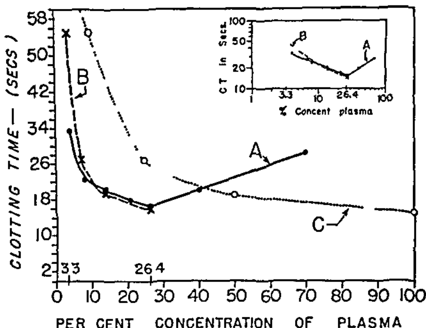


FIGURE 43

CURVE A The effect of adding a strong thromboplastin to stable normal plasma at different concentrations of the plasma in the clotting mixture Total volume of each clotting mixture 0 3 ml. throughout Silicone technique used up to the point when the plasmas were placed in glass clotting tubes Plasmas derived from oxalated human blood (9 ml. blood plus 1 ml of 0 1 M Sodium oxalate) Concentration of the thromboplastin the same throughout

CURVE B Curve plotted from values obtained by testing the same plasma according to the Quick one stage prothrombin time" method Total volume of the mixture 0 3 ml Same concentration of thromboplastin as that used for values in curve A The points on this curve as well as on curve A represent the actual con centration of the plasma in the final clotting mixture

CURVE C The points on curve C are the same as those of curve B but are plotted according to the common practice of referring plasma concentration not to the actual clotting mixture but to the alleged original concentration of the plasma without regard for dilution by the anticoagulant and the other clotting reagents It is the familiar dilution curve used for calculation of prothrombin concentration" by the one-stage method

the sense that it has heretofore been an unexplored area It is obviously an area of uncertainty results of work in this area are easily affected by the mode of collection of the blood its content of first phase inhibitors the type of surface used and the duration of contact of the plasma with it It was this uncertain performance of the plasma at concentrations of even 10 percent that drove Dr Link[3] to seek even lower concentrations namely 3 3 percent

centrations between 10 percent and 30 percent Asbestos plasma from which much of the action of first phase inhibitors is removed, is hypercoagulable even in high concentrations and its clotting time gradually lengthens as the plasma is diluted Whether on simple arithmetic or logarithmic ruling the curves for hemophilic and normal plasma follow a parabolic course while those for asbestos plasma follow the hyperbolic course If the plasmas are tested at the concentrations used in the Quick one stage method (26 4%) the differences between hemophilic and normal plasma are slight but those between normal and asbestos plasma are substantial At 3 3 percent concentration (that used in the Link Shapiro prothrombin time method) the differences in clotting time between the three plasmas are not significant It may be stated that the three plasmas contained the same amount of prothrombin and Ac globulin measured by the two stage method[1][2] The asbestos plasma resembles in many of its characteristics plasma that has lost much of its stability after being collected and stored in glass tubes

In Figure 43 are contrasted the curves expressing the relation between plasma concentration and activated clotting time when due attention is paid to the final concentration of the plasma in the clotting mixture (curves A and B) or when concentration of the plasma is stated (as is the general custom) as that of the original plasma (curve C) When the curve for dilution of plasma tested by the one stage prothrombin time method is plotted with due attention to the final percent plasma concentration of each clotting mixture the curve B almost superimposes a segment of curve A begun at high plasma concentrations (70 percent) Curve B therefore when correctly plotted is only a part of curve A mainly its ascending segment It begins at 26 percent concentration, or at the bottom of the valley of curve A That is the reason why curve B seems hyperbolic and to require only two constants in its empirical formula When the dilution curve is begun from higher plasma concentrations it takes a parabolic course an empirical formula to fit curve A or similar parabolas of the second order requires at least three constants It can now be seen how far these two curves differ from curve C which is the familiar dilution curve for the one stage method allegedly expressing the relation between concentration of plasma (or of prothrombin) and activated clotting time in seconds The one stage method covers the response of plasma to thromboplastin only from plasma concentrations of 26 percent or below The area of the curve to the right of this point may be called the No Man s Land of the one stage method in

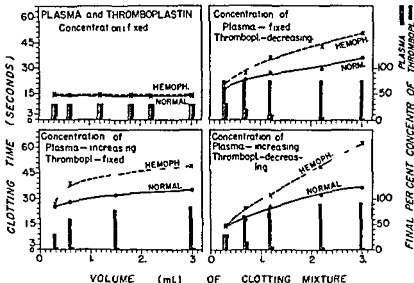


FIGURE 44

Effect of changes in the concentration of the plasma and thromboplastin in the final clotting mixture on the activated plasma clotting times (indicated by crosses and dots) of stable normal and hemophilic plasma collected with the silicone tech nique and tested in silicone-coated tubes Concentrations of the plasma and thromboplastin (represented by vertical bars) in each clotting mixture are expressed in terms of percent respectively of the original plasma and thromboplastin solutions

about and the greatest differences between the two plasmas are seen

Figure 45 illustrates the response to rabbit brain thromboplastin of three types of plasma tested at two concentrations The hyper coagulable plasma was obtained from a patient after hemorrhage and transfusion The three plasmas had normal content of prothrombin and Ac globulin (two stage method) The clotting times of all three plasmas at 26 percent concentration were shorter than at 70 percent even though the amount of thromboplastin and the total volume of the two mixtures were the same While no dif ference was demonstrable between the clotting times of the three plasmas at 26 percent concentration when 0 02 mg or more of thromboplastin were used at 70 percent plasma concentration there was a difference from the start which became accentuated as progressively smaller amounts of thromboplastin were added At 70 percent concentration hemophilic plasma failed to clot when it received amounts of thromboplastin (0 0018 mg 0 00018 mg)

(usually referred to as 12.5 percent) and may have influenced Dr. Smith[4] to employ a plasma concentration of 0.5 percent in the prothrombin conversion phase of his two stage method. It was clear to most of those working in the field that the clotting time did not truly and consistently begin to vary inversely as the prothrombin concentration until the plasma was diluted 1 part in 15 or more before being tested. Testing at lower dilutions often produced what seemed to be an anomalous behavior: the clotting time of 1:2 diluted plasma might come out to be the same or even shorter than that of undiluted plasma.

We may say, therefore, that high concentrations of plasma in a clotting mixture slow its response to thromboplastin, though such mixtures obviously contain greater amounts of prothrombin, Ac-globulin and fibrinogen than more diluted plasma mixtures. It can also be shown that this effect is especially striking in hemophilic plasma. When the plasma concentration is relatively low (about 30 percent) and the thromboplastin high, the response of hemophilic and normal plasmas is usually alike, regardless of the volume of the clotting mixture (Figure 44, left upper square). Whether the total volume of the mixture is 0.3 ml. or 3 ml., the clotting time is the same (15 secs.) provided the concentrations of plasma and thromboplastin are maintained constant. This came as somewhat of a surprise to us, accustomed as we were to thinking of volume as an important variable in the rate of coagulation. On inert surfaces like silicone coatings, total volume does not seem to be as important a variable as it is on glass surfaces. Moreover, differences that have been attributed to volume variation have perhaps been caused by inadvertent changes in plasma concentration in the clotting mixtures. We regret to say that it is at this lower level (about 30 percent) of plasma concentration that most tests are conducted designed to show that no differences exist between the response of normal and hemophilic plasma to thromboplastin, or that normal plasma has a clot accelerating action on hemophilic plasma.

If the concentration of the plasma is increased while that of thromboplastin is kept constant (Figure 44, lower left square) two changes are noted: a) the clotting time rises and b) the response of the hemophilic plasma becomes slower than that of the normal. A similar response is observed when the plasma concentration is maintained high and constant but that of thromboplastin is gradually reduced (Figure 44, right upper square). Finally, when plasma concentration is increased and thromboplastin decreased (Figure 44, right lower square) a summation of the two effects is brought

plasma represents of course the usual one stage prothrombin time mixture so convenient and informative in some respects but also unfortunately so misleading in others

Employing plasma at high concentrations makes it possible to test the response to thromboplastin of plasmas of various grades of stability Plasma from a severe hemophilic may require to be diluted 20 fold before its clotting time equals that of the normal and a dilution of 100 fold before approaching its own clotting time at 91 percent plasma concentration (Figure 46 curve C)

The stability of normal plasma and its resistance to activation by thromboplastin can be magnified even above that of the hemophilic by adding to normal plasma the lipid antithromboplastin separated from brain powder discussed before this Conference last year[5] In this manner unusually stable plasmas are obtained which when in high concentration strongly resist activation by thromboplastin but like hemophilic plasma lose this property rapidly on dilution (Figure 47) The now familiar parabolic curves are

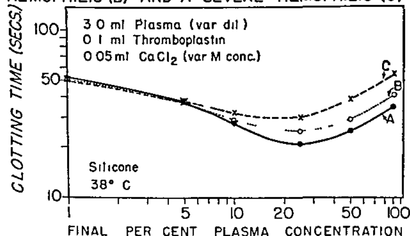


FIGURE 46

Note that when a large volume of plasma and a relatively weaker concentration of thromboplastin is used that a significantly different response to thromboplastin may be observed between a normal plasma and a plasma from a mild and a severe hemophilic When sufficiently diluted (plasma concentration of 5%) the three plasmas respond alike to thromboplastin.

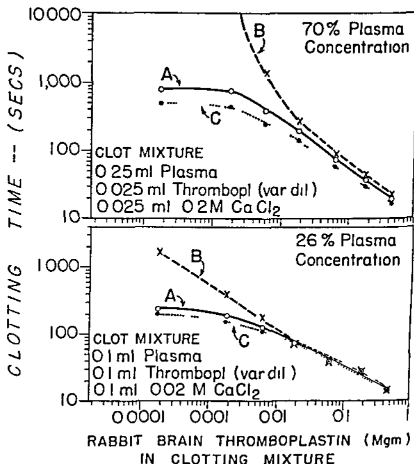


FIGURE 45

The plasma was handled by the silicone technique up to the time it was placed in glass tubes for testing Rabbit brain thromboplastin prepared from acetone dried rabbit brain Blood collected by the silicone technique (9 ml blood plus 1 ml 0 1 M Sod oxalate)

which easily clotted the same plasma at 26 percent concentration Similarly at the higher plasma concentrations differences were manifest between the normal and hypercoagulable plasma that were small or nonexistent in the 26 percent plasma The 26 percent

of plasma (in this and other determinations) whether done de liberately or otherwise has removed much of the action of natural inhibitors which therefore have escaped being observed As a general rule slightly or nondiluted plasma affords the best medium for the study of inhibitors while the opposite applies to coagulants such as prothrombin

It has been observed often that the rate of coagulation of citrated or oxalated recalcified plasma is shorter than that of whole blood from which the plasma is derived The reason is that the rate of blood coagulation is measured on undiluted blood while that of the plasma is done on specimens diluted by the addition of anticoagulant and recalcifying solutions In most tests involving recalcified plasma the final concentration of the plasma in the clotting mixture is between 30 percent and 60 percent This added to the fact that such plasma has before being tested been exposed to glass during collection and centrifugation more than accounts for the finding that recalcified citrated plasma though platelet poor clots faster than its parent blood If the silicone technique is used in collection of the blood separation and testing of the plasma and attention is paid to maintaining plasma concentrations in the final mixture of 70 percent or above it is not difficult to demonstrate that recalcified citrated plasma (platelet poor) will often not clot at all or take much longer to do so than the blood from which it is derived

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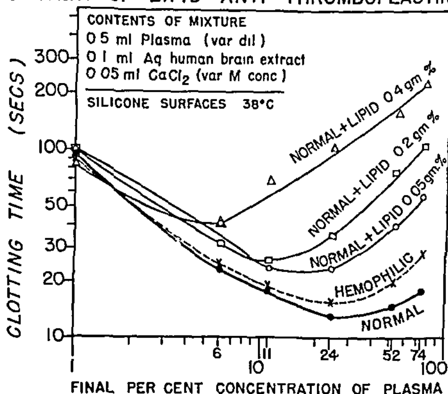


FIGURE 47

The lipid antithromboplastin was separated from human brain powder homogenized in a supersonic chamber and added to the plasma in the amounts indicated Prothrombin (1) and antithrombin (6) content of the plasmas were not altered by the lipid

made even more evident when the lipid inhibitor is added to the plasmas The greater the content of the inhibitor the more prominent is the descending right limb of the curve The more concentrated the plasma is in the clotting mixture the greater the difference between individual plasmas The less concentrated the plasma is in the clotting system the less difference between the plasmas which originally show widely different rates of coagulation This is why most prothrombin estimation methods have been more reliable if done on diluted plasma since in such methods the effect of the inhibitors is felt less or not at all By the same token dilution

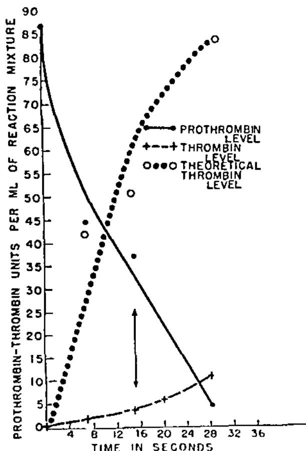


FIGURE 48

up to the moment of clotting only 1/14 of the thrombin ever be comes effective

Since the antithrombin effect was so striking we repeated our analysis to determine if 12.5% dilution of the plasma prior to the Quick test would minimize the activity of antithrombin Figure 49 shows this experiment Again the upper curve represents the pro thrombin titer the lower curve is the thrombin actually present and the middle curve the amount of thrombin theoretically formed if none had been inactivated The Quick test on the diluted plasma gave a clotting time of 30 seconds as is indicated by the arrow Examination of the curves at the time of theoretical clotting shows

THE IOWA TWO STAGE ANALYSIS OF THE QUICK PROTHROMBIN ASSAY USING 12 5 PERCENT SALINE DILUTED PLASMA

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At the Second Blood Clottmg Conference our presentation on the *Conversion of Prothrombin* contamed an analysis of the Quick one stage prothrombin assay In this experiment we used the re action mixture recommended by Dr Quick These reagents gave a clotting time of 15 seconds You will recall that in our experiment we repeated the test many times using the identical reagents with the exception that the plasma had been defibrinated At selected intervals, samples were removed from the reacting mixture for two stage prothrombin and thrombin assays

Figure 48 shows the same data presented at the First Conference but replotted to show the actual prothrombin and thrombin units in a milliliter of the reaction mixture As Dr Tocantins indicated in doing the Quick test on so called whole plasma there is about a 3 5 fold dilution of the plasma in the final clotting tube This 3 5 fold dilution includes the original addition of anticoagulant to the blood and the subsequent addition of the calcium and thrombo plastin If the whole plasma had 300 units of prothrombin per milli liter the reaction mixture used in the Quick test will have approxi mately 90 units per milliliter The lower curve of Figure 48 repre sents the thrombin titer, as determined experimentally The upper curve is the prothrombin titer and the middle curve is the total amount of thrombin that would have been present if none had been inactivated The arrow on the chart indicates the theoretical clotting time of 15 seconds It will be noted that at the time clotting oc curred only 4 units of thrombin were actually present Yet when we examine the upper curve we see that 55 units of prothrombin had disappeared If none of the converted prothrombin had been inacti vated the actual thrombin level would have corresponded to the middle curve As we pointed out last year the Quick test measures only a fraction of thrombin formed— a fraction so short lived that

Of interest is the apparent stability of the thrombin titer after clotting The chart shows that for 30 seconds the thrombin titer remained relatively stationary Experiments carried for longer periods of observation indicate that after 60 seconds the titer gradually falls It seems that with dilute plasma there is a brief interval during which the fall is slow

Similarly it is of interest to note that with both dilute plasma and whole plasma clotting occurs when the amount of prothrombin converted was somewhat less than half and in both cases the thrombin titer was 4 units The experiments also show that when the concentration of thrombin gradually increases from zero to 4 units the effect on fibrinogen is no greater than when a smaller amount of thrombin acts continuously for the same length of time The difference between thrombin forming and thrombin already formed seems reasonable since in a Quick test there are a few seconds in which the total thrombin unitage is less than 1 unit and a few seconds in which the thrombin is formed immediately prior to clotting

DISCUSSION

Alexander How did you do the thrombin assays?

Flynn A sample of the reaction mixture was removed and added to sodium citrate solution to stop the conversion of prothrombin to thrombin The citrated thrombin solution was then added to oxalated or citrated plasma and the clotting time noted The thrombin units in the reaction mixture could be calculated from the clotting time so obtained and the dilution factors of the thrombin

Alexander I am bothered by the experiments cited by Dr Astrup two years ago in the First Conference of this Group when he spoke of a certain chicken experiment

Flynn You mean A Fischer s famous experiment (Coagulation of blood as an unlimitedly transferable chain reaction *Biochem Ztschr 279, 108 (1935)*)

Alexander It is intriguing to me that you were able to fish out an aliquot and stop further prothrombin conversion to permit you sufficient time to quantitate the amount of thrombin evolved

Flynn We have tried to repeat Fischer s experiment using a different source of thromboplastin All our efforts failed but of course by using a different source of thromboplastin we were altering the method of Fischer

Quick The point which Dr Flynn brought out is very important namely that in the one stage method the time at which the clot

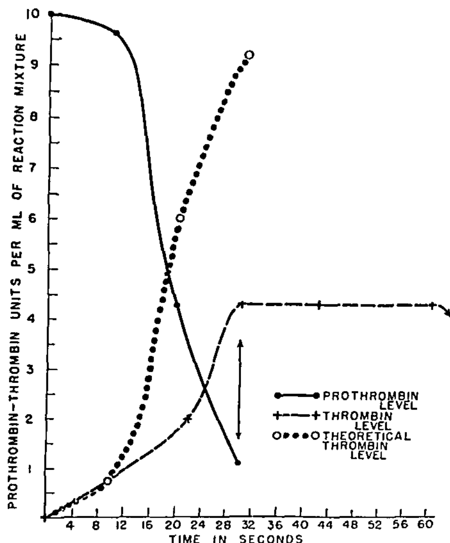


FIGURE 49

that 9 units of prothrombin had been converted to thrombin but the thrombin titer was only 4 units It is apparent that the discrepancy between the amount of thrombin present and the amount actually formed is still marked but less in the case of diluted plasma In the unmodified Quick test 93% of the total amount of thrombin formed is inactivated at the time of clotting With the diluted modification of the Quick test 60% of the thrombin has been inactivated when clotting occurs

Tocantins These are all collections by silicone technique

Quick But you finally transfer your material to glass?

Tocantins Some of the specimens yes

Quick I would suggest that the experiment be repeated using native plasma in which no anticoagulants have been added and probably you will find that "No Mans Land" of the one stage method becomes a fairy land of the one stage method

Tocantins That *was* done with hemophilic native plasma The only dilution entailed at first then is that caused by addition of the thromboplastin solution The same parabolic curve results

Quick That is very interesting I shall attempt to repeat that because if it is true it is a very important experiment

forms is not a measure of the complete conversion of prothrombin to thrombin In other words, in this reaction we measure the speed of the reaction and when a certain amount of thrombin is produced a clot is formed Fundamentally we are all working with the basic reaction that thrombin reacts with fibrinogen and the speed with which the fibrinogen is clotted is a function of the concentration of thrombin I think you will all agree with that statement In the one stage test, perhaps little prothrombin is actually converted to thrombin at the moment the clot forms, but the more prothrombin *is present, the faster the minimum* amount of thrombin required for the clot is formed The clotting time therefore remains a measure of the prothrombin concentration In this connection it might be interesting to mention our study of the prothrombin consumption in clotted blood Dr Faver Gilley and I allowed blood to clot and to remain in the water bath for an additional 15 minutes We then covered the unretracted clot with a layer of sodium citrate to pre vent any further reaction The serum obtained after centrifugation had nearly as much prothrombin as the original plasma In other words even though the blood had completely clotted so little pro thrombin was consumed that it could not be measured Of course as soon as clot retraction occurs the chain reaction is set off and even during the short time required to obtain serum by centrifuga tion enough prothrombin is converted to thrombin to cover the fact that the complete coagulation occurred with only a minute conver sion of prothrombin to thrombin Only after we found the trick of covering the clotted blood with sodium citrate were we able to demonstrate this I believe that little prothrombin is consumed when a clot forms intracorporally

I enjoyed the interesting observations which Dr Flynn made and presented In regard to Dr Tocantins work I would like to suggest that he repeat some of his work on native plasma kept in silicone Many years ago at the Fifth Avenue Hospital here in New York I did an experiment similar to those of Dr Tocantins I took normal and hemophilic blood put 1 cc of each in separate test tubes and to each added 0 1 cc of thromboplastin The coagulation time was not any different in the hemophilic blood which showed that the prothrombin concentration in hemophilia is normal and that no anticoagulant is present

Tocantins Collected in silicone?

Quick No it was not collected in silicone since it did not exist at the time the experiment was done The blood was collected in an ordinary syringe and transferred immediately to a glass test tube

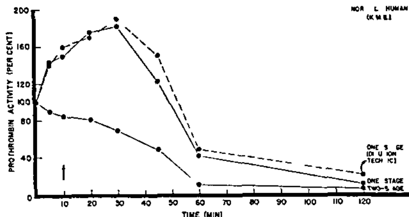


FIGURE 50

Two-stage tests were made both with and without added beef serum no great differences in results were observed and only the former are plotted One-stage tests were of two types in one procedure labelled "one-stage" in the chart, the clotting mixtures consisted of 0 1 ml thromboplastin, 0 1 ml. BaSO treated plasma 0 2 ml 0 02 M CaCl and 0 1 ml. citrated test sample In the other procedure labelled "dilution technic" in the chart, the clotting mixtures consisted of 0 01 ml. citrated test sample diluted 1 10 with BaSO treated plasma 0 1 ml thromboplastin and 0 01 ml $CaCl_2$. Clotting times by the one-stage test were converted to percent by use of so-called "dilution curves For values shorter than the control, two procedures were followed (a) the test sample was diluted 1 2 or 1 3 with BaSO treated plasma prior to doing the test and appropriate correction made in the percentile values (b) the clotting time was converted directly to percent by use of an equation similar to that described by Quick (Quick A J The clinical significance of prothrombin as a factor in hemorrhage *Penn Med J 43 125 (1939)*) The arrow indicates clotting time (Lee-White)

The results of one experiment with hemophilic dog blood[2] are shown in Figure 51 The slow loss of prothrombin by the two stage

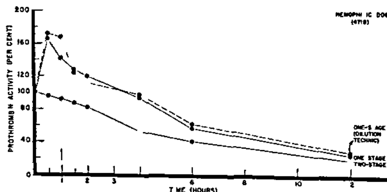


FIGURE 51

THE PROBLEM OF PROTHROMBIN DETERMINATIONS IN SERUM*

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We have been interested in doing simultaneous prothrombin determinations by both the one and two stage methods on clotting blood and serum A few individual experiments will illustrate the procedure and results Figure 50 shows the results obtained with normal human whole blood The blood clotted in 10 minutes Periodically, before during and after clotting citrate solution was mixed with the blood samples to stop the clotting process Determinations were done promptly on the supernate obtained by centrifugation The two stage test shows a progressive loss of prothrombin The one stage tests on the other hand show a period of hyperactivity which begins soon after the blood is drawn and which persists for about 50 minutes The peak of hyperactivity is reached at about 30 minutes when the value is about 180 percent Even after the values fall below 100 percent it will be noted that the one stage results are consistently above those with the two stage method

Somewhat similar results have been observed with normal dog blood except that the prothrombin disappears more rapidly by the two stage method and the hyperactive period by the one stage method is of shorter duration (25 minutes) with a peak of 180 percent at about 10 minutes

Experiments of this same type were performed on platelet poor human plasma and on hemophilic dog blood With the platelet poor plasmas[1] a slow but consistent loss of prothrombin was observed with the two stage test after the first hour In one experiment, a hyperactive phase was observed throughout the 8 hour period of observation with a peak value of about 200 percent This plasma contained approximately 3000 platelets per cu mm

* This investigation was supported in part by a research grant from the Division of Research Grants and Fellowships of the National Institutes of Health U S Public Health Service

is that added plasma is not going to make much difference in these results

We have adsorbed and eluted the prothrombin in serum using barium sulphate and sodium citrate somewhat similar to that done by Dr Alexander Such serum prothrombin products are hyper active also

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DISCUSSION

Smith I presume Dr Brinkhous your serum did not have much thrombin left

Brinkhous We originally thought that the serum thrombin content would explain many discrepancies between the two methods We repeatedly have done thrombin assays on the serum by merely omitting the calcium and thromboplastin in the one stage test mixtures With dog serum no clots formed in 10 to 15 minutes

Smith The little thrombin present in serum was inactivated by

procedure is shown, while again the one stage method shows a hyperactive phase preceding the slow decline to low values These data are of interest in relationship to Dr Quick s results on hemo philia In 1947 he modified our prothrombin utilization test, using the one stage test on serum by supplying a separate source of fibrinogen[3] From his results he concluded that there was a quantitative lack of prothrombin conversion in hemophilia and that our earlier results[4] were erroneous in this regard One can see how Dr Quick might reach such a conclusion by comparing the 4 hour prothrombin determinations by the two types of procedures the one stage values are approximately 100 percent while by the two stage procedure roughly half of the prothrombin had been lost

It is obvious from these results that the determination of pro thrombin in serum is a different matter than its determination in plasma attention has been called to this fact[5] We all recognize that serum from the standpoint of clotting factors is very much different than plasma the fibrinogen is gone prothrombin decreases and I think we have good evidence that the antihemophilic activity is lost[6] Furthermore there is the appearance and then loss of thrombin — the so called thrombin tide — a term popularized by Dr H P Smith[7] There apparently is a tide also of serum Ac globulin[8] Other accelerators may become apparent and there appear to be variations in antithrombin We must look I suppose to these differences in plasma and serum for an explanation of the divergent results obtained in these experiments by the two methods It is obvious that the two tests are measuring something different While the results of the two stage test appear to be dependent on the prothrombin content of the serum the one stage test appears to measure the balance that exists between (a) the changes in serum which tend to increase the convertibility rate of prothrombin and thus to shorten the prothrombin time and (b) the actual prothrombin level which as it decreases tends to prolong the prothrombin time

It has recently been suggested by Owren[9] that analyses of serum for prothrombin are not valid because of loss of a hitherto un recognized clotting factor present in plasma Dr Flynn suggested privately that this factor should be called Factor VII to be in line with Dr Owren s previous nomenclature If plasma containing this new factor were added to serum then one should recover from the serum prothrombin in amounts comparable to that originally present in plasma We do not We did not know about Owren s report when we did this work so it will have to be reexamined My impression

fusion of huge amounts of normal plasma to hemophiliacs we were never quite able to get the coagulation time in glass down to the mean value obtained on normal individuals It is extremely rare if at all that the clotting time of a hemophilic in glass can thus be brought to the mean normal value I know Dr Quick would like to discard the clotting time as a clinical test I too would like to discard it because it is subject to enormous error I think Dr Tocantins has pretty well shown that by progressively increasing the concentration of plasma in his system that he is approaching the clotting time in glass If one extrapolates the concentration from 70 percent to 90 percent and 100 percent it merely reflects a clotting time in glass with the added stipulation that he provides thromboplastin

We have also run an experiment with freshly drawn hemophilic blood promptly mixed in glass with normal blood of a homologous blood group drawn simultaneously I suppose that would be com parable to Dr Tocantins experiment where we considered the endothelium a "silicone lined vessel" and immediately removed blood from that "silicone lined vessel" and put it in glass We have found that even mixing equal proportions of normal blood with hemophilic blood did not restore the clotting time to the clotting time of the normal individual. The mixture required 2-3 minutes longer to clot than did the normal.

Allen What is your normal clotting time?

Alexander The mean normal clotting time of 100 subjects by the Lee and White technique at body temperature is 7 7 minutes with a standard deviation of 1 7

Allen I think we are missing a real opportunity to study blood coagulation by not pursuing blood as a whole The work of Dr Tocantins reinforces this view While it is true that the prothrombin time depends upon a rapid acceleration of recalcified plasma using a potent thromboplastic material this procedure overcomes the effects of any minor inhibitor that might influence the first phase of coagulation This can be demonstrated when a dilute thromboplastin material is used in the presence of heparin

The whole blood clotting time as we perform it is essentially a five tube modification of the original one tube Lee White procedure Blood is drawn into a syringe that has been coated with paraffin This blood is immediately placed into a dry chemically clean and unscratched test tube from which we aspirate 5 cc of blood into a graduated pipette One cc is then placed in each of 5 soft glass test tubes That would seem to be an excessive manipula

antithrombin and in the presence of citrate no more thrombin was formed

Brinkhous We did the tests for thrombin as quickly as possible after citration About the shortest time that we could centrifuge and manipulate the material to make the test was 4 minutes

Smith By adding fibrinogen it does not clot?

Brinkhous That is a different story Here we were using adsorbed plasmas, which contain a lot of antithrombin, as the source of fibrinogen The serum does cause purified fibrinogen to clot We did not believe a test for thrombin with purified fibrinogen would be a proper control to determine the extent thrombin might be the cause of the hyperactive phase

Alexander Did you take normal serum obtained hours after clotting and add that to oxalated plasma and see what you would get?

Brinkhous No We were interested in this study in the hyper active phase which occurs relatively early

Alexander I can state that in our experience Dr Brinkhous observations on hemophilia and thrombocytopenia — this is arti ficially induced thrombocytopenia — are perfectly valid and in agreement with observations we have reported recently (Alexander B and Landwehr G Prothrombin consumption serum prothrombic activity and prothrombic conversion accelerator in hemophilia and thrombocytopenia *J Clin Investigation* 28 *1511 (1949)*) There is a hyperreactivity of the prothrombin in thrombocytopenic serum and hemophilic serum and also in silicone serum or in serum ob tained from blood allowed to clot in the presence of heparin which will retard coagulation So all these observations are valid by the one stage procedure whereas you indicate that by the two stage procedure there is a progressive although retarded drop in the prothrombin content of the serum I would like to come back to Dr Tocantins observations which I think are of great interest from the point of view of trying to unravel the explanation for the clotting defect in hemophilic blood I think it is exceedingly important because the question has always remained is hemophilia referable to excessive amounts of anticoagulant inhibiting clotting in the first phase or is it due to deficiency of a plasma component essen tial to clotting?

I would like to cite some observations that we have made (Alexander B Landwehr G Studies of hemophilia I The control of hemophilia by repeated infusions of normal human plasma *J A M A* 138, *174 (1948)*) where even after the intravenous in

time to be done right is too troublesome for most laboratories to undertake and that unless it is done well it is better not done at all Nevertheless it is the only tool we have for the measurement of the clotting qualities of blood as a whole,

Tocantins If anything should be thrown out it is the technique of collection of the blood There is nothing wrong with the measurement of the rate of coagulation of the blood if carefully observed with properly collected specimens placed in properly prepared tubes

Allen With the technique that we employ in our laboratory silicone is not only unnecessary but undesirable With silicone coated equipment the normal clotting time runs in the neighborhood of three hours

Barker I would like to ask Dr Ferguson to discuss one phase of the original subject the two stage prothrombin test We have had a good many discussions about variations in results of the one stage test when different thromboplastins are used in different laboratories I have previously regarded the two stage test as not subject to such variations but I have wondered if the results were always comparable I understand that Dr Ferguson has some data on this subject which may indicate that the two-stage test also is subject to variability

tion of blood, however with venipuncture technique perfected the clotting time ranges between 25 and 40 minutes and under these circumstances prolongations of the clotting time can be detected that otherwise go unnoticed Admittedly, the procedure is time consuming and is not suitable for any technician or physician who is not willing to spend the time required to overcome the sources of error By this procedure we can pick up the effect of quantities of heparin approximately 1/5th that which are normally declared necessary to produce a prolongation of the clotting time

Barker It is not clear to me what you put in the tube besides the blood

Allen Nothing

Mann Heparin?

Allen No With this technique the clotting time for the dog ranges between 25 and 35 minutes and for man it ranges between 25 and 40 minutes

Tagnon At what temperature?

Allen Room temperature I presume ours might be a little more shortened if it were done at body temperature but in the summer time when it gets hot in Chicago there is not too much difference

Tagnon It is an entirely new thing to me to hear that blood would clot in 25 to 40 minutes under these circumstances I cannot explain it by the temperature effect I wonder if somebody has another explanation for it

Alexander I have been interested in one of the modifications in the technique and that is the syringe used to draw the blood is coated with vaseline

Allen I might say we get very little difference if we have a syringe with no coating whatever

Alexander You use a tube which is 1 mm in diameter?

Allen It is very important that you use the same type all the time

Alexander In the orthodox Lee White method the soft glass tube is 100 by 11 mm in diameter We use the 2 cc and of course there is a greater surface area in relation to the size of the tube and all those things make a difference

Allen They make a difference There is no question about that

Alexander They all have to be taken into consideration I too would love to throw out the clotting time and discard it but at the moment —

Quick You have my permission

Allen I agree with Dr Quick that the whole blood clotting

at each step in the procedure in order routinely to obtain assay data reliable to within an error limit of about 10 percent Ware and Seegers detailed directions[3] are splendid Our reagents exceed their minimal standards particularly in the case of fibrinogen thromboplastin serum (for which we determined an optimal concentration of 1 300 in the serum saline diluent) and acacia (we use a Ca and oxalate free purified powder containing less than 0 4% NaCl)

Fibrinogen Like Ware and Seegers we find Armours Bovine Fibrinogen (Fraction I) which Dr Mann has used successfully[4] in the Mayo Clinics modification of the two stage test to give assay values which are apt to be rather too low and too variable for recommended routine use There are evidently certain factors relating to the reactivity of fibrinogen which are still needing investigation We did find the cold fibrinogen of Ware Guest and Seegers[5] generally satisfactory provided we added an adsorption with $BaSO_4$ in order to reduce the prothrombin content which otherwise proved troublesome in our preparations

Control tests with prothrombin free fibrinogen A 1 percent solution of Armours Bovine Fibrinogen (in borate buffer ph of 7 7) adsorbed once or twice with $BaSO_4$ until free from all traces of prothrombin and already lacking any thrombin or fibrinolysin impurities is an excellent reagent for controlling a) thrombin b) prothrombin c) antithrombin d) fibrinolysin in serum thromboplastin and plasma Confidence in our reagents and procedures was strengthened by the addition of such control tests

II ASSAY RECOVERY OF PURIFIED PROTHROMBIN

We found (in confirmation of the experience of the workers with purified prothrombin) that Seegers highly purified prothrombin a preparation which had been kept in dry powdered form for over a year in a dessicator at room temperature first had lost about half of its original potency that second it was unstable in solution depending on temperature but not fully controlled even in frozen solutions stored at –20 C and that third it lacked thrombin in fresh solutions but gained an appreciable contamination with thrombin in several days at room temperature or longer periods at refrigerator temperatures In unexplained discrepancy with the data we reported last year[6] but confirming the findings reported by Dr Ware and Dr Seegers[7]* the unitage of this spontaneous thrombin formation as determined by the two stage method failed

* Also by personal communication

THE TWO STAGE PLASMA PROTHROMBIN ASSAY SOME STUDIES PERTAINING TO ITS TECHNIQUE AND RESULTS OF ADDING PURIFIED PROTHROMBIN, HEPARIN AND FIBRINOLYSIN

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Owing to the exigencies of time, I must limit presentation of my ideas to a few highlights It seems to me that the key question as to what these alleged prothrombin tests really measure can be focussed in some specific ways Due to the splendid work initiated under Smith, Warner and Brinkhous at Iowa and continued in Detroit by Seegers and colleagues we now have highly purified prothrombin Why have we not heard a great deal more about the ability of the various assay tests to show quantitative recovery of purified prothrombin added to plasma systems? Surely predictable recoveries, under these circumstances would be the strong est kind of evidence that the tests really are measuring prothrombin In 1941 we obtained assay increments very close to the determined activity of crude (Howell type) prothrombin added to plasma which had been diluted 50 to 100 times(1)

The technique used then and throughout the many years of our *in vitro* clotting studies, relies upon the significance of varying clotting times as a measure of relative thrombin potency It is a different principle which is employed in the modern two stage method namely to use varying dilution and conclude that a fixed clotting time (15 seconds under the standardized conditions) repre sents an absolute thrombin unit We discussed some of the implica tions of these differences at the 1949 meeting(2) This year one of my graduate students C L Johnston, Jr has become proficient in the use of the two stage assay procedures and contributes the following data

I THE TWO STAGE PLASMA PROTHROMBIN ASSAY

Technical Matters A neophyte would seem to require some months of hard effort to become thoroughly familiar with the pre paring control testing and standardization of the reagents and in checking optimal concentrations time factors and conditions

TABLE XXXI

EFFECTS OF VARIOUS CONCENTRATIONS OF HEPARIN UPON CLOTTING TIMES AND THROMBIN UNITS OBTAINED IN FINAL STEP OF THE TWO-STAGE ASSAY OF PLASMA PROTHROMBIN

	1	2	3	4	5	6
HEPARIN CONCENTRATION u/ml final clotting mixt)	0	0 134	0 26	0 4	0 66	6 6
CLOTTING-TIME (seconds)	15 6	17 4	17 9	18 8	20 0′	28 8
ESTIM THROMBIN UNITS (per ml "original plasma)	191	166	161	152	144	88

TABLE XXXII

EFFECTS OF VARIOUS CONCENTRATIONS OF HEPARIN ADDED AFTER DEFIBRINATION UPON CLOTTING TIMES AND THROMBIN UNITAGE IN TWO STAGE ASSAY OF PLASMA PROTHROMBIN

	1	2	3	4	5	6	7
HEPARIN CONCENTRATION 1 (u/ml thrombic mixt) 2 (u/ml final clot mixt)	 0 0	 0 02 0 016	 0 168 0 134	 0 33 0 26	 0 67 0 536	 0 75 0 6	 1 25 1 0
CLOTTING TIME (seconds)	15 6	15 2′	100″	135′	164	194	212
ESTIM THROMBIN UNITS (per ml "original" plasma)	191	194	–	–	–	–	–

TABLE XXX

ASSAY RECOVERY OF PURIFIED PROTHROMBIN (Pro) ADDED TO PLASAMA (H Pl) BEFORE (I) AND AFTER (II) DEFIBRINATION IN TWO STAGE METHOD OF DETERMINING PROTHROMBIN ACTIVITY

	Mixture	Incubation Time (Min)	Total Found (units/ml)	Total Expected (units/ml)	% Deviation from Expected
A	H Pl + Pro	16	228	227	0
B	H Pl + Pro	22	160	144	+11
C	H Pl	14	188	–	–
D	Pro	35	99	–	–

to account for most of the loss in prothrombin activity I shall not reopen this problem but merely state the practical conclusion that it is essential to determine the actual prothrombin in any solution of the purified reagent at the time of performing the following recovery experiments

Data Average of triplicate determinations by the modified two stage test are summarized in Table XXX for the following A) 0 5 ml human plasma + 0 4 ml purified prothrombin (0 02 percent) added *before* defibrination with 0 1 ml 100 unit per ml thrombin B) 0 5 ml plasma (diluted 1 2 after usual defibrination) + 0 5 ml prothrombin C) plasma (alone) D) prothrombin (alone) Note that the assay recoveries are as expected in A and practically at the upper limit of experimental error in B a very satisfactory answer to the question of assay recovery

III EFFECTS OF HEPARIN ON RESULTS OF THE TWO STAGE ASSAY

A *Added in second phase (thrombin fibrinogen reaction)* Using various amounts of heparin in the saline by which our stock fibrinogen (*analyzing 1 8 percent 1 5 percent protein and 85 percent* 90 percent clottable) was diluted to 1 0 percent before testing the clotting times with the (approximately) 1 unit of thrombin optimally formed in the diluted activated plasma processed by the usual modified two stage method we obtained the data summarized in Table XXXI The results show how sufficient heparin (acting no doubt in conjunction with such cofactor as is presumably supplied in the reagents) influences the clotting times and thrombin units obtained the latter being calculated as usual per ml of original

heparin was present during the defibrination It would therefore appear that something occurs in the defibrination procedure which can vary the assay result especially in the presence of physiological amounts of heparin which are too small to show any of the first or second phase inhibitory actions on the clotting system as modified by the steps of the two stage procedure

IV EFFECTS OF FIBRINOLYSIN

A year or two ago Dr E C Loomis supplied us with a dried preparation of bovine serum fibrinolysin of good potency upon which we have reported[10] So far we have tested only a limited range of fibrinolysin concentrations (0 0067 0 075 mg per ml incubation mixture) in both the second and first phases of coagulation as in the foregoing experiments with heparin The data fail to show any significant variations in plasma prothrombin unitage by the modified two stage assay method Fibrinogenolysis was not a factor in these experiments and at the enzyme concentrations used there was no acceleration of thrombin formation of clotting times or fibrinolysis for many hours

CONCLUSIONS

The general conclusion from these studies is that there are a number of variables under empirical control in the two stage method about which we do not yet have sufficient information but merely suggestive data to indicate that they may at times become significant As our experimental knowledge increases we may expect to gain in understanding of what the unitages obtained really mean in terms of factors other than prothrombin which although it is the major determinant of assay values may not be the only factor to influence the test results

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plasma, uncorrected for hematocrit The smallest heparin concen tration tested definitely prolongs the clotting time and lessens the computed thrombin unitage

B *Added in first phase (activation of prothrombin to thrombin)*

1 *Heparin added AFTER defibrination* In the data of Table XXXII the heparin was added (a) with the serum saline diluent in tests 2 5, (b) with the incubation mix in test 6, and (c) immediately after the defibrination in test 7 The least amount of heparin (0 02 u per ml in thrombic mixture) had no significant effect but all higher concentrations tested (0 168 1 25 u per ml) greatly prolonged the clotting times caused clots of poor quality (flocculent and stringy) and made determination of the thrombin unitage impossible The optimal incubation periods were only slightly increased however It is particularly significant that 0 168 u per ml heparin (test 3) prevented assayable thrombin formation although the second phase effects (Table XXXI test 2) were very minor

2 *Heparin added BEFORE defibrination* When 0 5 ml plasma was mixed with 0 4 ml heparin (33 u per ml) before defibrination to correspond with the above (0 168 u per ml) heparin concentra tion in the final (1 200) clotting mixture no thrombin was detectible in the incubation mixture for many hours Such amounts of heparin of course are far in excess of physiological concentrations such as may be reached in anticoagulant therapy Accepting the figure of 0 001 mg (0 1 unit) per ml of whole blood as a typical value in anticoagulant therapy[8][9] we approximated this by adding 0 1 u per ml heparin to plasma during the 1 2 dilution immediately *before* defibrination (i e 0 5 ml plasma + 0 4 ml heparin (0 25 u per ml) + 0 1 ml thrombin) the rest of the assay procedure being continued in the usual way In 4 preliminary experiments with similar amounts of heparin added *after* the defibrination the average per ml unitage (range in parentheses) was 194 (184 202) In 12 controls without heparin similar values of 201 (192 212) were obtained In 12 experiments heparinized before defibrination as stated the assay values were 233 (224 240) that is significantly elevated in every case

This unexpected result poses some new and difficult problems which have so far eluded our attempts at a satisfactory solution We did find however that the assayed unitage in a purified pro thrombin solution (81) *un*altered by the addition of 0 1 ml (5 units) of thrombin was increased to 89 when 0 2 ml fibrinogen was added (with 0 1 ml thrombin) and to 100 when 0 1 unit

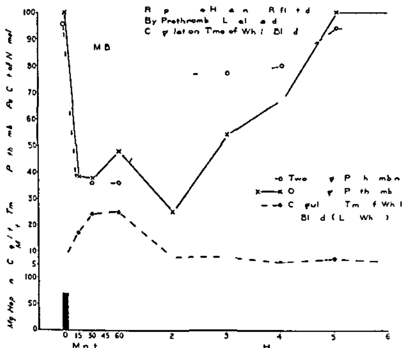


FIGURE 52

One other point for the possible benefit of some of you who have been using the two-stage test in the Ac globulin assay we in the last two weeks have found that if we adapt part of Dr Owren s technique in his Factor V assay namely if we collect human plasma with oxalate and allow it to stand for a matter of 10 or 12 days the accelerator factor as he mentioned disappears and that plasma then will give us a very satisfactory curve for Ac globulin We use 25 cc of a 2 5 percent potassium oxalate solu tion and add up to 250 cc of whole blood collected as we collect it for our blood bank We allow it to stand at 4 C until the one stage prothrombin time reaches more or less infinity i e over 100 seconds We siphon off the plasma assay for prothrombin and Ac globulin using the two stage method and then we divide it into half cc portions and store it at –35 This method I should like to say is subject to all the criticisms that the method we have been discussing here are subject to namely that we have un doubtedly a lot of factors there that we are not accounting for and it may give us variables but we get curves such as Dr Seegers

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DISCUSSION

Olwin In regard to the point that Dr Ferguson made we have studied in some patients the effect of heparin given intravenously on both the one stage and two stage methods and at the same time on the Lee White coagulation time of whole blood in this instance using the one tube technique (Figure 52 from Archives of Surgery *58, 603 May 1949*) You can see that a 60 mg dose of heparin intravenously showed a definite lowering of the prothrombin 10 minutes later of shall we say the prothrombin as measured by the 2 tests This effect lasted for a period of 1 hour and then gradually there was a return to normal by the end of 6 hours Whereas we get this type of curve in the one stage test showing that the effect is prolonged (which we interpreted guardedly as the influence of heparin on more than one of the coagulation factors and should I say possibly anticoagulation factors) the coagulation time as done on this patient showed an effect lasting for 1 hour and then a return to pre heparin levels at the end of 2 hours On the basis of this finding we use the one stage test as a measure of the effect of heparin therapeutically and as a control for its dosage

Two other points I would like to make which are not related to the last one We have used a number of Armour s fibrinogen products and none of them has been satisfactory until recently Within the last 6 months they have made a product which is quite uniformly reactive If put into solution and stored at —35 it remains normally reactive though its activity diminishes slowly over a period of a week or 10 days However it can be used very satisfactorily for the two stage test if made up each day or every few days

has shown you with his Ac globulin assay Those of you who have been using the test are familiar with the curves They very closely parallel the ones that we have obtained with some of Dr Seegers purified products and I just mention it as another tool that some of us may use

Overman I have two questions that I would like to ask Dr Tocantins In our work on the thromboplastin inhibitor we find that the calcium chloride concentration is very important in controlling the activity of the inhibitor and also the time factor of just standing in a glass tube (Overman R S The chemical purification and mode of action of a thromboplastin inhibitor *Blood Clotting and Allied Problems* J E Flynn Ed Trans Second Conf New York Josiah Macy Jr Foundation 1949 (p 177))

I was wondering whether with these high concentrations of plasma (74 percent) you can bring the clotting time of the normal plasma down to the adsorbed plasma by increasing the calcium ion concentration or by setting up your experiment and letting it stand for an hour or so and then running your test?

Tocantins We have not been able to do it by raising the calcium concentration But by allowing the plasma to stand in contact with glass the coagulability of the plasma increases until the clotting times of the higher plasma concentrations approach those of "adsorbed" plasma It may take from 2 to 3 hours of contact with glass and intermittent stirring of the plasma in the glass container to bring about that effect At all times in these experiments optimal concentrations of calcium were used

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